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Investigations of the relationship between infections with *Borrelia burgdorferi* and glomerulonephritis in Bernese Mountain dogs

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Habilitation

Originally published at:

Gerber, B. Investigations of the relationship between infections with *Borrelia burgdorferi* and glomerulonephritis in Bernese Mountain dogs. 2010, University of Zurich, Vetsuisse Faculty.

Vetsuisse-Fakultät der Universität Zürich

Klinik für Kleintiermedizin

(Direktorin: Prof. Dr. Claudia Reusch)

**Investigations of the relationship between infections with
Borrelia burgdorferi and glomerulonephritis in Bernese
Mountain dogs**

**(Untersuchungen zum Zusammenhang von Infektionen mit
Borrelia burgdorferi und Glomerulonephritiden beim Berner
Sennenhund)**

Habilitationsschrift

Zur Erlangung der Venia Legendi der Vetsuisse-Fakultät der Universität Zürich

vorgelegt von

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Zürich, 2010

Content

Chapter 1	Introduction and Goals	5
Chapter 2	Increased prevalence of <i>Borrelia burgdorferi</i> infections in Bernese Mountain Dogs: a possible breed predisposition	15
Chapter 3	Association of urine protein excretion and infection with <i>Borrelia burgdorferi</i> sensu lato in Bernese Mountain dogs	25
Chapter 4	Follow- up of Bernese Mountain dogs and other dogs with serologically diagnosed <i>Borrelia burgdorferi</i> infection: What happens to seropositive animals?	29
Chapter 5	Complement C3 in Bernese Mountain dogs	39
Chapter 6	Comparison of a rapid immunoassay for antibodies to the C6 antigen with conventional tests for antibodies against <i>B. burgdorferi</i> in dogs in Europe	47
Chapter 7	The dilemma with Lyme borreliosis in the dog with particular consideration of „Lyme nephritis“ [Das Dilemma der Lyme Borreliose beim Hund unter besonderer Berücksichtigung der „Lyme Nephritis“. In German]	53
Chapter 8	Summary and Conclusions	61
Chapter 9	Acknowledgments [in German]	67

Chapter 1 Introduction and Goals

Preamble

Is there a causal relationship between Lyme disease (borreliosis) and glomerulonephritis in Bernese Mountain dogs? The question arose because Bernese Mountain dogs had a high incidence of glomerular kidney disease and were often found to be positive in tests for antibodies against *Borrelia burgdorferi* as well (Preiss, 1991; Reusch et al., 1991; Minkus et al., 1994; Reusch et al., 1994).

Borreliosis in dogs

Borrelia are spirochetes with a worldwide distribution. Transmission from one animal to another is via ticks of the *Ixodes* genus. Species belonging to the *B. burgdorferi* sensu lato (henceforth referred to as *B. burgdorferi* for brevity) complex are the cause of Lyme disease or borreliosis, and in humans lead to clinical signs involving the skin, joints, meninges and cardiac muscle. These signs are typically preceded by “erythema migrans“, a characteristic circular erythematous rash associated with the tick bite. In Europe, the most important species belonging to the *B. burgdorferi* complex are *B. garinii*, *B. afzelii* and *B. burgdorferi* sensu stricto. *B. burgdorferi* sensu stricto is the primary species seen in the USA.

Dogs are also susceptible to Lyme borreliosis. Young beagles, experimentally infected with *B. burgdorferi*, had fever, intermittent lameness with swollen joints, lymphadenopathy and anorexia (Greene and Straubinger, 2006). However, “erythema migrans“ was not seen. Postmortem examination 49 to 581 days post infection revealed enlarged lymph nodes with follicular and parafollicular hyperplasia, polyarthritis that was associated with swollen joints in some dogs, dermatitis in the region where the tick was attached, and in some dogs, periarteritis and perineuritis in the joint capsule, perinodal soft tissues and skin. Lesions were not seen in any other organs (Summers et al., 2005). Other signs that have been associated with borreliosis in dogs, such as rheumatoid arthritis (Roush et al., 1989), neurological dysfunction (Azuma et al., 1993), cardiac arrhythmia (Levy and Duray, 1988) and glomerulonephritis (Dambach et al., 1997), could not be reproduced in experimental studies.

Bernese Mountain dogs and glomerulonephritis

Glomerulonephritis is a renal disease that manifests as protein loss through the urine and a decrease in the functional mass of the kidneys (Vaden, 2005). The most important sequels are edema, effusion and renal failure. Deposition of immune complexes, which result from chronic infection, is the most common cause of glomerulonephritis.

In a study at the University of Zurich, it was shown that the incidence of glomerulonephritis in Bernese Mountain dogs was 13.9 times higher than in the general dog population (Preiss, 1991). An increased incidence of glomerulonephritis in Bernese Mountain dogs was seen by numerous clinicians and confirmed in other studies (Reusch et al., 1991; Minkus et al., 1994; Reusch et al., 1994). The Bernese Mountain dogs described in those studies had membranoproliferative glomerulonephritis with interstitial nephritis, which was usually fatal. The dogs at the same time had high antibody titers to *B. burgdorferi*. This led to the question of a possible causal relationship between *B. burgdorferi* infection and glomerulonephritis in Bernese Mountain dogs.

Borreliosis and glomerulonephritis

A putative association between Lyme borreliosis and glomerulonephritis was discussed as early as 1987 (Magnarelli et al., 1987). Five dogs with antibodies to *B. burgdorferi* developed renal disease during or after episodes of intermittent lameness. The renal disease was not characterized further but was associated with proteinuria and hypoalbuminaemia in some of the dogs. Spirochete bacteria identified as *B. burgdorferi* were detected in the kidney tissue of one dog. In a report one year later, glomerular lesions were described in a Labrador Retriever with a high antibody titer to *B. burgdorferi* (Grauer et al., 1988); *Borrelia* were identified in the renal interstitium using immunofluorescence. In the 1990s, two studies of 49 dogs with antibody titers to *B. burgdorferi* and specific renal lesions (Dambach et al., 1992; Dambach et al., 1997) were published. Most of the lesions were membranoproliferative glomerulonephritis, diffuse cortical tubular dilatation with tubular necrosis and tubular regeneration as well as diffuse interstitial, lymphoplasmacytic inflammation. The term „Lyme nephritis“ was coined, although a direct relationship between Lyme borreliosis and glomerulonephritis had not been established (Littman et al., 2006).

Complement C3 glomerulonephritis and *B. burgdorferi* infection

The complement system is an important part of innate immunity and together with acquired immunity provides defence against infection, including infection with *B. burgdorferi* (Aberer, 2007). Activated complement and specific antibodies are responsible for opsonisation and lysis of bacteria. Some species of *B. burgdorferi* are able to evade complement-facilitated elimination, possibly through binding of complement-inhibiting factors such as factors H and FHL-1 (factor H-like protein 1), which inhibit the production of complement C3b and enhance its inactivation (Alitalo et al., 2001). Infection can be established by bacteria that evade the complement system. *B. afzelii* has been shown to bind complement-inhibiting factors, whereas *B. garinii* has been considered very sensitive to complement-facilitated elimination. *B. garinii* is the most common species of *Borrelia* in Switzerland (Jouda et al., 2003). A deficiency in complement could theoretically result in a marked increase in infection with *B. garinii* because of a lack of complement-facilitated elimination.

Complement C3 is the most important component of the complement system (Tizard, 2004). Dogs with a genetic deficiency of complement C3 have a high incidence of renal disease with glomerulonephritis (Blum et al., 1985). Of 20 affected dogs, five had clinical signs of membranoproliferative glomerulonephritis and 14 of the remaining 15 had histological renal lesions consistent with membranoproliferative glomerulonephritis (Cork et al., 1991). Complement has been shown to play an important role in immune complex-mediated renal disease (Berger and Daha, 2007). In a study of children with type II membranoproliferative glomerulonephritis, there was a decreased level of complement C3 in most of the patients (Schwartz et al., 2001). In 88% of the children, antibodies to complement C3 convertase, the so-called C3-nephritic factor, were found. This factor leads to extension of the half-life of complement C3 convertase and thus, to a deficiency of complement C3. Therefore, a deficiency of complement C3 can be initially involved with glomerulonephritis as well as with *B. burgdorferi* infection.

Diagnosis of Lyme borreliosis

„Erythema migrans“ is the hallmark of Lyme borreliosis in humans (Stanek and Strle, 2003). However, because this sign does not occur in dogs, clinical manifestation of the

disease is much less clear (Littman et al., 2006). To complicate matters, there are no standardised tests in dogs (Jacobson et al., 1996). Diagnosis of *B. burgdorferi* infection is usually based on detection of antibodies using a combination of ELISA or indirect immunofluorescence and Western blot (Littman et al., 2006). However, different tests and interpretations of the tests are responsible for considerable variations of the results. Culture and polymerase chain reaction (PCR) are other ways of identifying *B. burgdorferi*, but those two methods have disadvantages in comparison with serological methods. Culture depends on specialised laboratories, is very labour intensive and often unproductive (Stanek and Strle, 2003). Polymerase chain reaction is easier to conduct but the specificity of identifying an active infection is considered low because the test does not differentiate between living and dead organisms (Hengge et al., 2003). False positive results are common when ELISA is used because of the similarity of antigens of *B. burgdorferi* to those of other spirochetes and other bacteria that may occur as commensals or pathogens in dogs.

In newer test methods, the specificity of ELISA has been increased by using the recombinant peptide C6 as the antigen. This peptide corresponds to IR6, an immunodominant region of the VlsE (Vmp-like sequence, Expressed) superficial lipoprotein of *B. burgdorferi*, and is genetically, structurally and antigenically conserved across various species of *B. burgdorferi* (Greene and Straubinger, 2006). It was first isolated from a European strain of *B. garinii*. The genes of IR6 are only expressed during replication in mammalian hosts. The peptide does not occur in ticks that have not had a blood meal, and therefore serves as a marker of active infection. The use of this peptide in serological tests is progressively replacing older methods. In the USA, tests based on the C6 peptide are considered to be highly specific (99%) for the diagnosis of borreliosis in humans. The sensitivity varied from 62% to 100% depending on the stage of the disease (Liang et al., 1999). A C6 ELISA proved to be reliable in detecting borreliosis in humans with clearly-defined clinical signs of Lyme disease in Europe (Liang et al., 2000a) although it was earlier found that European patients had a lower antibody response to infection with *B. burgdorferi* (Dressler et al., 1994; Hauser et al., 1997).

ELISA tests based on the C6 peptide have also been used in dogs. In an American study, a C6 ELISA produced the same results as a whole-cell ELISA combined with Western blot (Levy et al., 2002). In experimentally-infected dogs, the antibody titer to C6 increased after infection more rapidly than titers measured with other methods, and decreased after

treatment faster than the antibody titers measured with other methods (Liang et al., 2000b; Straubinger et al.; Philipp et al., 2001). Compared with other tests, the C6 ELISA test does not cross react with dirofilaria, babesia, ehrlichia or leptospira and does not produce positive results in dogs that have been immunized against *B. burgdorferi* (Liang et al., 2000b; O'Connor et al., 2004). The C6 ELISA test has been used in dogs in Europe (Krupka et al., 2007), but has not been compared with conventional tests. In the USA, *B. burgdorferi sensu stricto* is the only species of *Borrelia* that causes Lyme borreliosis, whereas in Europe, the disease is usually the result of infection with *B. afzelii* or *B. garinii* (Steere, 2001). Therefore, although the C6 ELISA is reliable for the diagnosis of borreliosis in dogs in the USA, the same could not automatically be assumed for dogs in Europe.

Problems and Goals

Based on information that was available, it was not clear whether there is an association between infection with *B. burgdorferi* and glomerulonephritis in Bernese Mountain dogs, or if the two occurrences are merely coincidental. To investigate this problem, the prevalence of antibodies to *B. burgdorferi* in Bernese Mountain dogs was first determined. Subsequently, Bernese Mountain dogs seropositive for *B. burgdorferi* were examined for an increase in proteinuria as a marker of glomerular disease and were monitored for signs of glomerulonephritis later in life.

Furthermore, because it was not known whether a deficiency in complement C3 is associated with *B. burgdorferi* infection and glomerulonephritis in Bernese Mountain dogs, the concentration of complement C3 was determined in *B. burgdorferi*-seropositive and seronegative Bernese Mountain dogs, and the results were compared with those of dogs of other breeds.

The reliability of the C6-ELISA test for diagnosis of Lyme borreliosis in dogs in Europe was investigated by comparing the results with those of the recommended combination of a conventional whole-cell ELISA with a Western blot.

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Chapter 2

Increased prevalence of *Borrelia burgdorferi* infections in Bernese Mountain Dogs: a possible breed predisposition

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BioMed Central Veterinary Research 2007, 3:15

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Contributions of B. Gerber:

B. Gerber designed the study, analyzed the data and drafted the manuscript.

Research article

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Increased prevalence of *Borrelia burgdorferi* infections in Bernese Mountain Dogs: a possible breed predisposition

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Published: 12 July 2007

Received: 22 November 2006

BMC Veterinary Research 2007, 3:15 doi:10.1186/1746-6148-3-15

Accepted: 12 July 2007

This article is available from: <http://www.biomedcentral.com/1746-6148/3/15>

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Abstract

Background: Glomerulonephritis in dogs has been associated with *B. burgdorferi* infections. In Bernese Mountain Dogs with glomerulonephritis antibodies against *B. burgdorferi* have been found in most dogs, raising the question if the breed is predisposed to infections with *B. burgdorferi*. The aim of this study was to determine the prevalence of antibodies against *B. burgdorferi sensu lato* in a well defined population of Bernese Mountain Dogs and to compare this prevalence with data from dogs of other breeds.

Results: 160 Bernese Mountain Dogs and 62 control dogs (large breed dogs with long hair) were included. All dogs were considered healthy according to a questionnaire filled out by the owner, complete blood count, chemistry panel, urinalysis and urine culture. Bernese Mountain Dogs and control dogs were kept in similar environments. Seroprevalence of *B. burgdorferi* was assessed by ELISA and Western blot and was 58% in Bernese Mountain Dogs compared to 15% in control dogs. This difference was significant. Neither antibodies against leptospires nor vaccination or hair coat color influenced the results.

Conclusion: The cause of the considerably higher prevalence of antibodies against *B. burgdorferi* in Bernese Mountain Dogs and its consequences are not known. A breed predisposition can be suspected.

Background

Glomerulonephritis in dogs has been associated with *B. burgdorferi* infections [1-5] and in some studies spirochetes were detected in the kidneys [2,3] and the urine [2]. However some of the authors questioned the relationship of a renal lesion with *B. burgdorferi* [1,3]; still others assumed *B. burgdorferi* to be the causative agent for renal lesions [2]. In Bernese Mountain Dogs, a familial glomerulonephritis was reported [4,5]. However, antibodies

against *B. burgdorferi* were found in most dogs, raising the question of whether the occurrence of glomerular disease in Bernese Mountain Dogs is related to an infection with *B. burgdorferi* or if the breed is predisposed to infections with *B. burgdorferi*.

The aim of this study was to determine the prevalence of antibodies against *B. burgdorferi sensu lato* in a well defined population of Bernese Mountain Dogs and to compare

this prevalence with data from dogs of other breeds from a similar environment.

Results

Dogs

One hundred and sixty Bernese Mountain Dogs and 62 control dogs were included in the study. Age, gender, hair coat color and breed are depicted in Table 1. Bernese Mountain Dogs were significantly younger than the control dogs ($p = 0.01$). Gender distribution was the same in both groups ($p = 0.41$). Fifty-six of the 62 control dogs belonged to 8 different long haired large breeds. The remaining 6 dogs were mixed-breed dogs with Collie, German Shepherd and Flat-Coated Retriever as dominant breeds.

The geographical distribution of the places where the dogs lived is depicted in (Figure 1).

The evaluation of the replies given to the questionnaires are depicted in Table 2. Analysis of the answers only revealed significant differences between the groups for the frequency of attached ticks. Significantly more Bernese Mountain Dog owners (44%) answered yes to the question whether the dogs often had attached ticks compared to owners of control dogs (25%; $p = 0.01$). The significance disappeared if only dark haired control dogs ($n = 20$) were compared with Bernese Mountain Dogs even though the percentage remained the same (25% and 44% respectively; $p = 0.08$).

The answers to the questions about the environment in which the dogs lived are depicted in Table 3. Significant differences were found between dogs which lived in a rural or a urban environment and for the percentage of time spent in the woods. A significantly larger number of

Bernese Mountain Dogs (95%) lived in rural areas compared to control dogs (79%; $p = 0.001$). Looking at the two groups separately, living in rural areas did not lead to a higher prevalence in antibodies against *B. burgdorferi* compared to an urban environment. The reported percentage of time spent in the woods during walks was significantly higher in Bernese Mountain Dogs with antibodies against *B. burgdorferi* compared to those without them ($p = 0.049$). In control dogs no significant difference was found ($p = 0.90$).

Antibodies against *B. burgdorferi*

In 160 Bernese Mountain Dogs and in 61 control dogs antibodies against *B. burgdorferi* were determined with both an ELISA and a Western blot. Of the Bernese Mountain Dogs, 92 (58%) had a positive ELISA with a positive Western blot, while in the control dogs this only happened in 9 (15%) dogs. This difference was significant ($p < 0.001$). In positive dogs ODs ranged from 0.21 to 2.00 (median 0.75) in negative dogs from 0.04 to 1.28 (median 0.18) (Figure 2). The ODs of positive Bernese Mountain Dogs were significantly higher than those of positive control dogs ($p < 0.001$). Seropositive dogs had 1 to 7 bands in the Western blot (median 4) while seronegative dogs had 0 to 3 bands (median 0) (Figure 3). The serology results are summarized in Table 4. Control dogs with a dark coat had significantly more antibodies against *B. burgdorferi* (28%) when compared with control dogs with a fair coat (7%; $p = 0.03$). Bernese Mountain Dogs whose owners reported frequently attached ticks did not have antibodies against *B. burgdorferi* significantly more often (69%) compared to Bernese Mountain dogs whose owners reported infrequently attached ticks (55%; $p = 0.07$). Control dogs whose owners reported frequently attached ticks did not have antibodies against *B. burgdorferi* significantly more often than control dogs whose

Table 1: Breed, age, gender and hair coat color of dogs included in the study

Breed	number of dogs	Age ¹ [years]		Gender [number of dogs]				hair coat color
		Range	Median	f	fs	m	mn	
Bernese Mountain Dogs	160	1-11	4	94	21	35	10	dark
Landseer	28	1-12	5	16	2	8	2	fair
Newfoundland	12	3-8	6	9	1	2	0	dark
Flat-coated Retriever	8	1-7	1	4	0	3	1	dark
Golden Retriever	3	1-5	4	3	0	0	0	fair
Saint Bernard	2	2/5	3.5	1	1	0	0	fair
Belgian Sheppard	1	2	2	1	0	0	0	fair
Mastin de los Pirineos	1	11	11	0	0	1	0	fair
Tibetan Mastiff	1	5	5	0	0	1	0	fair
Mixed breed dogs	6	2-8	6.5	0	1	1	4	5 fair, 1 dark
Control dogs total	62	1-12	5	34	5	16	7	41 fair, 21 dark

f = female, fs = female spayed, m = male, mk = male neutered

¹Significant difference between Bernese Mountain Dogs and control dogs ($p = 0.01$).

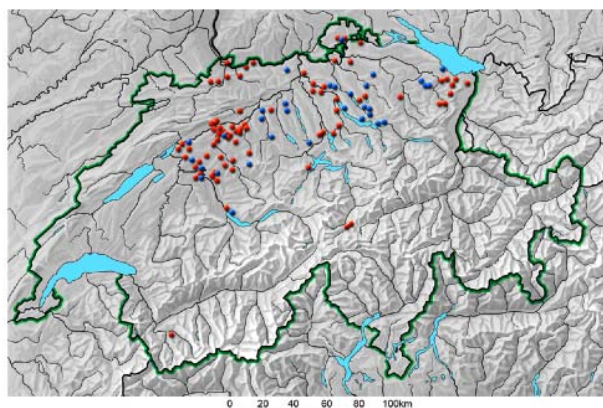


Figure 1
Map of Switzerland with the geographical distribution of tested dogs. Origin of Bernese Mountain Dogs (red dots) and control dogs (blue dots).

owners reported infrequently attached ticks (23% and 13% respectively; $p = 0.30$).

Comparison of antibodies against *B. burgdorferi* and against leptospires

Of 92 Bernese Mountain Dogs with antibodies against *B. burgdorferi*, 53 (58%) had antibodies against leptospires as well and of the 68 without antibodies against *B. burgdorferi*, 33 (49%) had antibodies against leptospires ($p = 0.16$). Of 9 control dogs with antibodies against *B. burgdorferi* there were 6 (67%) with antibodies against leptospires, and of 53 negative control dogs 28 (53%) had antibodies against leptospires ($p = 0.35$).

Antibodies against *B. burgdorferi* and vaccination against Lyme borreliosis

Four Bernese Mountain dogs and 6 control dogs had been vaccinated against Lyme borreliosis. All 4 Bernese Mountain dogs had a positive ELISA but only 3 had a positive Western blot. Of the 6 control dogs, 3 had a positive ELISA and a positive Western blot and 3 were negative on both tests.

Discussion

The higher prevalence of antibodies against *B. burgdorferi* in Bernese Mountain Dogs indicates a breed predisposition. Susceptibility in some breeds to a certain infection is known from other diseases. For instance Rottweiler, American Pit Bull Terrier, Doberman Pinscher, Pomeranian, and German Shepherd Dog are breeds at significantly greater risk for parvovirus enteritis than mixed breed dogs [6]. The reason is not known. However common ancestry has been associated with this. Intense breeding might

have led to a decrease in defense against infections. This might also be true for Bernese Mountain Dogs, a breed that is known for intense breeding and that has a narrow gene pool. This is supported by the fact that several diseases are prevalent in Bernese Mountain Dogs such as bleeding tendency, epilepsy, and malignant histiocytosis [7-11]. However no infections have been described so far. In Cavalier King Charles Spaniels a breed that was known to be prone to *Pneumocystis pneumonia* infections, it was found that there was a immunoglobulin deficiency in the affected dogs indicating defect in immunity in these dogs [12]. The findings in the present study are unique as infections with *B. burgdorferi* are not causing disease. Furthermore, no immunodeficiency is known in Bernese Mountain Dogs. At the time, *B. burgdorferi* was associated with glomerulonephritis in Bernese Mountain Dog, no direct relation to the disease could be made. It is possible that the Bernese Mountain Dogs with glomerulonephritis in this study had antibodies against *B. burgdorferi* because the over all prevalence of antibodies was so high in this breed [5]. Labrador- and Golden Retrievers were found to be overrepresented in a group of dogs with distinctive renal lesions attributed to Lyme disease and also among seropositive dogs in a survey performed in Texas [1,13], indicating some breed predilection for *B. burgdorferi* infections. However Bernese Mountain Dogs were not mentioned in the studies.

It is well established that *Borrelia* organisms evade the immune system in different ways and host factors become more important the less pathogen the responsible organisms are [14,15]. In human patients with Lyme disease-associated erythema migrans, the carriage rate of leukocyte class II alleles DRB1*0101 and DRB1*0101-DQB1*0501 was higher in patients with the least pathogenic *B. burgdorferi* genotype [15]. The immunologic event causing this association was not known but as DRB1 alleles are located close to certain major histocompatibility complex-encoded complement genes, it was speculated that variants of these complement genes might be in linkage disequilibrium with the DRB1 alleles [15,16]. The innate immune response plays an important role in the early response of *Borrelia* [17] but it also plays a role in the development of certain glomerular diseases. Dogs with a genetically determined deficiency of complement C3 more often develop renal and infectious diseases [18]. The occurrence of a complement disturbance would explain the co-occurrence of infection with *B. burgdorferi* and glomerular disease. However no such disturbance is known in Bernese Mountain Dogs so far.

In one study 5% to 34% of the *I. ricinus* ticks in Switzerland were infected with *B. burgdorferi* and infected ticks were found in all areas where ticks were collected [19]. These figures remained stable in later studies [20-25].

Table 2: Evaluation of replies to questions regarding health status of the dogs by questionnaire

		Bernese Mountain Dogs			Control dogs				
		<i>B. burgdorferi</i> serology		total	<i>B. burgdorferi</i> serology		total		
		positive	negative		positive	negative			
					hair color		hair color		
					fair	dark	fair	dark	
Does your dog often have attached ticks? ¹	Yes	40	18	58	1	2	7	3	13
	No	40	33	73	1	4	24	11	40
Do you perform tick prevention?	Yes	52	38	90	2	6	23	5	36
	No	26	14	40	0	0	7	7	14
Did your dog suffer from infectious diseases?	No	62	47	109	2	3	25	12	42
	Yes	15	4	19	0	3	5	2	10
General health	Normal	80	52	132	2	6	30	14	52
	Abnormal	0	0	0	0	0	0	0	0
Endurance	Normal	76	50	126	2	6	28	14	50
	Decreased	4	2	6	0	0	2	0	2
Weight loss	No	75	52	127	2	6	30	14	52
	Yes	3	1	4	0	0	0	0	0
Skin normal	Yes	69	48	117	1	6	28	14	49
	No	9	4	13	1	0	2	0	3
Appetite	Normal	72	50	122	1	6	30	14	51
	Decreased	5	2	7	0	0	0	0	0
	Increased	1	0	1	1	0	0	0	1
Thirst	Normal	72	52	124	2	6	29	14	51
	Decreased	5	0	5	0	0	1	0	1
	Increased	1	0	1	0	0	0	0	0
Vomiting	No	77	52	129	2	6	29	14	51
	Yes	1	0	1	0	0	1	0	1
Coughing	No	77	52	129	2	6	30	14	52
	Yes	0	0	0	0	0	0	0	0
Urine volume	Normal	74	52	126	2	6	30	14	52
	Decreased	1	0	1	0	0	0	0	0
	Increased	1	0	1	0	0	0	0	0
Defecation	Normal	76	51	76	2	6	30	14	52
	Diarrhea	2	1	2	0	0	0	0	0
Lameness	No	46	36	46	1	6	24	14	45
	Yes	5	3	5	1	0	6	0	7
Fever	No	80	53	80	2	6	30	14	52
	Yes	0	0	0	0	0	0	0	0
Edema	No	51	39	51	2	6	30	14	52
	Yes	0	0	0	0	0	0	0	0

¹Significant difference between Bernese Mountain Dogs and control dogs ($p = 0.01$).

Even though owners of control dogs reported more often that they lived in urban areas, the Bernese Mountain Dogs and the control dogs in the present study were kept similar, lived in the same areas of Switzerland, were walked in the same frequency and for the equal amount of time in the woods. In addition all tick-infested areas in Switzerland harbor infected ticks. Based on this Bernese Mountain Dogs did not appear to have a higher risk of tick exposure compared to control dogs. Furthermore in a Dutch study, prevalence of antibodies were not different between dogs considered at high risk to a *B. burgdorferi*

infection (hunting dogs) and those considered at low risk (pet dogs) [26]. One explanation for this was that the rate of outdoor walking in house dogs was considered higher and this also applies to Bernese Mountain Dogs or other large breed dogs in Switzerland. Nevertheless Bernese Mountain Dog owners in this study reported that their dogs had attached ticks more often than owners of control dogs and it is known that seropositivity among dogs is positively associated with increased tick exposure [27]. However the question whether the dogs had attached ticks more often was not specified and was therefore subject to

Table 3: Evaluation of replies to questions asked by telephone interview regarding the environment the dogs lived in

		Bernese Mountain Dogs		Control dogs	
		number of dogs	<i>B. burgdorferi</i> positive [%]	number of dogs	<i>B. burgdorferi</i> positive [%]
Was the area you lived in rural or urban ¹	Rural	136	59	37	11
	Urban	6	50	10	30
Did your dogs have access to the house or did he live only in a kennel?	Access to house	127	56	41	17
	Kennel only	16	81	6	0
Had your dogs access to a run?	Yes	136	60	45	16
	No	7	43	2	0
Could your dog escape from the house or the run?	Yes	28	50	4	0
	No	115	61	43	16
How often did you walk the dog a day?	1 time	68	62	18	11
	2 times	51	57	16	19
	3 times	20	50	8	0
	>3 times	3	67	5	40
What percentage (%) of time did you spend in the woods on your walks? ²	Range	0–100		0–100	
	Median	50		50	

¹Significant difference between Bernese Mountain Dogs and control dogs ($p = 0.001$).

²Bernese Mountain Dogs with antibodies against *B. burgdorferi* spent a significantly higher percentage of time in the woods on walks than those without ($p = 0.049$).

the individual judgment of the owner. Furthermore neither the Bernese Mountain Dogs nor the control dogs which had a high frequency of attached ticks reported, had a significantly higher prevalence of antibodies against *B. burgdorferi* compared to those which did not frequently have ticks.

A possible reason for the increased exposure of Bernese Mountain Dogs was the dark hair coat. In dark hair it is more difficult for the owner to detect ticks than in dogs with fair hair color. This would allow more time for the *Borrelia* species to move from the tick to the host and infect the dogs [28-30]. In the control dogs it could be shown that the ones with dark hair had significantly more often antibodies than those with fair hair (28% versus 7%). However, if dark haired control dogs were compared with Bernese Mountain Dogs, it could be seen that they

also had significantly less often antibodies against *B. burgdorferi* compared to Bernese Mountain Dogs even though statistically significant differences in reported tick exposure disappeared. This indicates that hair color is not the explanation for the higher seroprevalence of antibodies against *B. burgdorferi* in Bernese Mountain Dogs. Furthermore it was found that people with white clothing attracted more ticks than people in dark clothing in the same environment over the same time period, indicating that fair hair might even attract more ticks than dark hair [31].

Results of serologic tests are not consistent. The specificity of whole cell ELISAs is limited because of cross reactivity with other organisms [32]. Even though Western blot was performed for the confirmation of the ELISA results antibodies of leptospires were measured to rule out cross reac-

Table 4: Results of serologic testing for *B. burgdorferi*

Serology*	Bernese Mountain Dogs (n = 160)			Control dogs (n = 62)		
	number of dogs	%	number of dogs	%	coat color	
					fair	dark
positive ^{1,2}	92	58	9	15	3	6
negative	68	42	53	85	38	15
total	160	100	62	100	41	21

* ELISA and Western blot positive

¹Significant difference between Bernese Mountain Dogs and control dogs ($p < 0.001$).

²Significant difference between control dogs with fair and dark coat color ($p < 0.03$).

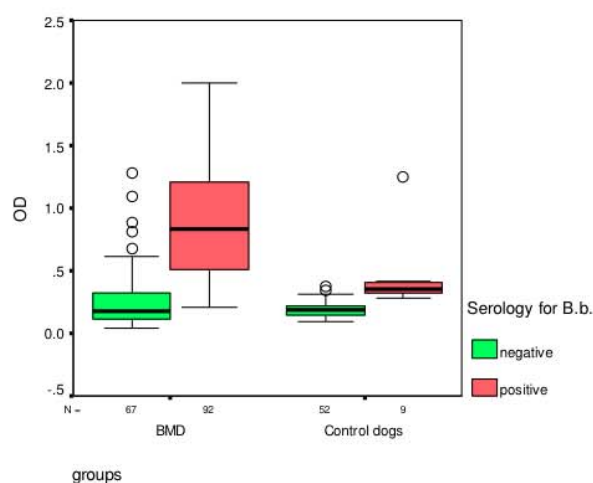


Figure 2
***B. burgdorferi* ELISA results (optical density 405 nm) from Bernese Mountain Dogs and control dogs.** OD = optical density, BMD = Bernese Mountain Dogs, B.b. = *Borrelia burgdorferi*

tion in the ELISA. Antibodies against leptospire are known to cross-react with antibodies against *B. burgdorferi* and it was found that antibodies against leptospire are common in healthy dogs in Switzerland [33,34]. Results of the microscopic agglutination test (MAT) for antibodies against leptospire showed that there was no influence of leptospire on the results of antibody tests for *B. burgdorferi*. Vaccination can influence ELISA results [35]. In Switzerland dogs are rarely vaccinated against *B. burgdorferi* and in the present study only 10 dogs were vaccinated, which did not seem to influence the study results.

There are different ways in which Western blot results can be interpreted [36]. In the present study criteria were used

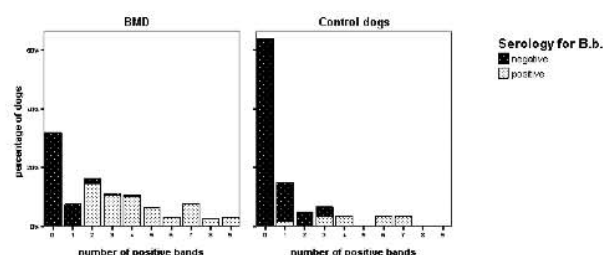


Figure 3
Number of positive bands depicted in a Western blot for *B. burgdorferi* from serum of Bernese Mountain Dogs and control dogs. BMD = Bernese Mountain Dogs, B.b. = *Borrelia burgdorferi*

that were established in Europe because European *B. burgdorferi* strains are different from strains in the USA. Also the antibody response of European human patients with Lyme Borreliosis was found to be variable and more restricted than that in U.S. patients [37,38]. Antibody response seemed stronger in seropositive Bernese Mountain Dogs compared to positive control dogs in this study as the reaction in the ELISA was stronger. Rather than differences in spirochete strains, in duration of the infection or in number of reinfections, differences in the immune response of the hosts are a possible explanation for this finding.

Conclusion

In conclusion this study showed that Bernese Mountain Dogs more often had antibodies against *B. burgdorferi* compared to control dogs. Breed predisposition for antibodies against *B. burgdorferi* has not been reported before. More investigations are needed to evaluate the biological reasons and consequences of infections with *B. burgdorferi* in Bernese Mountain Dogs.

Methods

Samples and dogs

The dogs whose owners belonged to the Club for Bernese Mountain Dogs in Switzerland were defined as the population to be examined. The number of dogs needed to predict the prevalence of antibodies in this population was calculated using the statistical software EpiInfo 6.1 (WHO, Genf, 1997). At least 131 Bernese Mountain Dogs were needed to predict an estimated prevalence of 10% with an accuracy of 5%. The prevalence of 10% was estimated according to prevalences found in the literature.

The minimum number of control dogs needed was calculated using the software WinEpiScope 2.0 (Nacho de Blas, Zaragoza, Spain, available online). The control dogs were to be long haired, large breed dogs resembling the Bernese Mountain Dogs in size and hair coats. The hair coat color of the control dogs was classified either as dark (similar to Bernese Mountain Dogs) or as fair.

Owners were contacted and volunteered to join the study after a call from the Swiss Club for Bernese Mountain Dogs and the Swiss Newfoundland and Landseer club. Others were directly contacted if it was known that they owned a dog eligible for the study.

Samples were collected between July 2002 and April 2003.

Dogs were included in the study if they were older than 4 months. The dogs were healthy according to the owners with no obvious signs of a specific disease evaluated by a complete blood count, a serum biochemical analysis and

urinalysis. Serum biochemical analysis included determination of bilirubin, glucose, urea, creatinine, total protein, albumin, cholesterol, sodium, potassium, chloride, calcium and phosphorus concentrations and measurement of the activity of alkaline phosphatase, alanine transferase, aspartate transferase and amylase. Urinalysis included urine test strip (Combur-Test®, Roche Diagnostics GmbH, Mannheim Germany), microscopic examination of urine sediment and determination of urine specific gravity and urine protein:creatinine ratio. The geographical area of Switzerland where the dogs originated was not previously determined and depended on the place where owners who wanted to join the study lived. Each owner was asked to complete a questionnaire and give information about the health status of the dog (Table 2). One specific question was whether the dog often had attached ticks. However the category "often" was not further specified.

The environment in which the dogs lived was investigated in retrospect by telephone interview (Table 3).

Serologic testing

An enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against *B. burgdorferi sensu lato* was performed in all dogs according to a method described earlier [39]. Briefly a whole cell sonicate of *B. burgdorferi sensu stricto* reference strain B31 (ATCC 35210) was used as antigen. The samples were previously absorbed with a heterologous sorbant consisting of washed formalin inactivated whole cells of *E. coli*, *Salmonella typhimurium*, *Brachyspira hyodysenteriae*, *Bacillus subtilis* and leptospires comprising 18 serovars. Western blot examinations for the detection of antibodies against *B. burgdorferi* were performed in all but one dog. A commercial test kit adapted for dogs was used (Virion Ltd., Rüschlikon, Switzerland). The tests which were performed according to the manufacturer's instructions consisted of Western blot strips with defined partial antigens of *B. burgdorferi ss.* and *B. afzelii*. In preliminary tests with positive and negative dog serum, a dilution of 1:200 and a conjugate (alkaline-phosphatase-rabbit-anti-dog IgG, H+L, Sigma, Diesenhofen, Switzerland) dilution of 1: 2000 was considered adequate. The interpretation of the Western blot results was done according to the interpretation criteria recommended for three European species of *B. burgdorferi sensu lato* [37]. Samples were considered positive if bands at the level of the partial antigens p100, p58, OspC, p21 or wb18 were identified or if at least two bands at the level of the partial antigens p45, bmpa und wb30 were present. Bands at the level of the partial antigens OspB, OspA, OspD, wb22 und OspE were considered unspecific.

For the microscopic agglutination test (MAT) to detect antibodies against leptospires, the ten most commonly

recognized serovars (sv.) in Switzerland were used as antigens: *Leptospira interrogans*, sv.: *australis*, *bratislava*, *autumnalis*, *bataviae*, *canicola*, *grippotyphosa*, *icterohaemorrhagiae* and *pomona*; *Leptospira borgpetersenii*, sv.: *hardjo* and *tarassovi*.

Statistical analysis

Data were recorded and analyzed using a commercial computer program (Statistical Package for the Social Sciences for Windows version 11, SPSS Inc., Chicago IL, USA). Between Bernese Mountain Dogs and control dogs variables were compared using the Mann-Whitney U test for the evaluation of age, optical density, percentage of time spent in the woods and a Fisher's exact test for all other data. Differences were considered significant at $p < 0.05$.

Authors' contributions

BG: Designed the study, analyzed the data and drafted the manuscript.

SE: Contributed to the study design, collected the data and contributed to the manuscript drafting and data interpretation.

MMW: Performed the serologic tests, was involved in the study design and the drafting of the manuscript

CER: Was involved in the study design and coordination and contributed to the critical evaluation and interpretation of the data.

Acknowledgements

We thank the owners who volunteered to join the study after a call from the Swiss Club for Bernese Mountain Dogs. This work was supported by the Swiss Club for Bernese Mountain Dogs, the Albert Heim Foundation and Novartis Animal Health Switzerland

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Chapter 3

Association of urine protein excretion and infection with *Borrelia burgdorferi* sensu lato in Bernese Mountain dogs

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The Veterinary Journal 2009; 182(3); 487-488

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Contributions of B. Gerber:

B. Gerber designed the study, analyzed the data and drafted the manuscript.



Short Communication

Association of urine protein excretion and infection with *Borrelia burgdorferi* sensu lato in Bernese Mountain dogsBernhard Gerber^{a,*}, Simone Eichenberger^a, Katharinan Haug^a, Max M. Wittenbrink^b, Claudia E. Reusch^a^a Clinic for Small Animal Internal Medicine, Vetsuisse Faculty University of Zurich, Winterthurststrasse 260, 8057 Zurich, Switzerland^b Institute of Veterinary Bacteriology, Vetsuisse Faculty University of Zurich, Winterthurststrasse 260, 8057 Zurich, Switzerland

ARTICLE INFO

Article history:

Accepted 28 August 2008

Keywords:

Bernese Mountain dog
Borrelia burgdorferi
Familial nephropathy
Lyme nephritis
Proteinuria

ABSTRACT

Bernese Mountain dogs (BMDs) are prone to develop a familial glomerulonephropathy and a pathogenic role of *Borrelia burgdorferi* sensu lato in this disease has been suspected. Glomerular disease in many affected dogs is clinically inapparent and proteinuria is found incidentally. In this study, urine protein excretion was evaluated in 122 clinically healthy BMDs and 55 controls. The seroprevalence of *B. burgdorferi* in BMDs was 57%, compared to 16% in controls. There were no significant differences in the occurrence of positive dipstick results, microalbuminuria, urine protein-to-urine creatinine ratio or abnormal urine protein pattern (determined by sodium dodecyl sulphate agarose gel electrophoresis) between BMDs and controls and BMDs with and without antibodies against *B. burgdorferi*. It was concluded that antibodies against *B. burgdorferi* are not associated with proteinuria as an early sign of renal disease in BMDs.

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Bernese Mountain dogs (BMDs) are prone to develop a familial glomerulonephropathy. Although pedigree analysis has indicated an autosomal recessive hereditary transmission, a possible pathogenic role of *Borrelia burgdorferi* sensu lato in the development of the disease needs to be taken into consideration, since all affected BMDs tested by Reusch et al. (1994) had antibodies against *B. burgdorferi*. Furthermore, the proportion of animals with antibodies against *B. burgdorferi* is significantly higher in healthy BMDs compared to healthy dogs of other breeds (Gerber et al., 2007).

A relationship between renal disease and infection with *B. burgdorferi* was suspected in previous studies, but a causative role of *B. burgdorferi* in the development of glomerulonephritis has not been confirmed (Magnarelli et al., 1987; Grauer et al., 1988; Dambach et al., 1997). Proteinuria is the hallmark of glomerular disease but many animals with glomerular disease are clinically inapparent and proteinuria is found incidentally during routine health screening. We hypothesised that healthy BMDs, in particular those with anti-borrelial antibodies, might show abnormal protein excretion as an early sign of glomerular disease. The aim of this study was to compare urine protein excretion measured by different methods between BMDs and dogs of other breeds and BMDs with antibodies against *B. burgdorferi* and those without antibodies.

Samples from healthy dogs used in a study of the prevalence of antibodies against *B. burgdorferi* BMDs were evaluated in this study

(160 BMDs and 62 control dogs) (Gerber et al., 2007). Only dogs in which urine protein excretion was measured by at least two of the following four methods were included in the evaluation: (1) Urine test strip (Combur-9 Test, Roche Diagnostics); (2) urine protein-to-creatinine ratio (UPC) measured colorimetrically with a pyrogallol red-molybdate-complex on a Cobas-Integra analyser (Roche), – dogs with UPC > 0.5 were considered to be proteinuric; (3) microalbuminuria, measured by a commercial rapid immunoassay for canine microalbuminuria (ERD Screen Test, Heska), where samples are diluted to a specific gravity of around 1.010 prior to albumin measurement and results are interpreted as negative, low positive, medium positive and high positive according to the manufacturer's instructions; (4) qualitative assessment of urine proteins by sodium dodecyl sulphate agarose gel electrophoresis (SDS-AGE); for this assay, a semi-automated system was used (Hydrasis Electrophoresis System with Hydragel 5 Proteinurie Agarosegel SEBIA) (Zini et al., 2004). Each urine sample (80 µL) was diluted with 20 µL of an additive provided by the manufacturer, then 5 µL of treated urine were loaded into the gel and the gel was placed into a device (Hydrasis) performing all phases of electrophoresis from migration to incubation to staining, destaining and drying. All gels were visually interpreted by the same person and were classified as normal if there were no bands or only a minimal band at 66 kDa (Zatelli and Bonfanti, 2002). A distinct band at 66 kDa and/or bands with higher molecular weights than 66 kDa were considered to represent glomerular proteinuria; bands with molecular weights below 66 kDa were considered to represent tubular proteinuria and the proteinuria was considered to be mixed if

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Table 1
Breed age and gender of dogs included in the study

Breed	Number of dogs	Age (years) ^a		Gender (number of dogs)	
		Range	Median	Female	Male
Bernese Mountain dog	126	1–11	4	87	39
Landseer	27	1–12	5	17	10
Newfoundland	9	3–8	6	7	2
Flat-coated Retriever	8	1–7	1	4	4
Golden Retriever	3	1–5	4	3	
Saint Bernard	2	2–5	3.5	2	
Belgian Shepherd	1	2	2	1	
Tibetan Mastiff	1	5	5		1
Mixed breed dogs	4	4–8	6.5	1	3
Total of control dogs	55	1–12	5	35	20

^a Significant difference between Bernese Mountain dogs and controls ($P = 0.027$).

Table 2
Results of tests for protein in the urine in Bernese Mountain dogs and control dogs

Test	Test results	Bernese Mountain Dogs (126)		Control dogs (55)	
		Antibodies against <i>B. burgdorferi</i>		Antibodies against <i>B. burgdorferi</i>	
		Yes (n)	No (n)	Yes (n)	No (n)
Dipstick	Negative	57	44	7	32
	1+ positive	12	10	2	13
	2+ positive	2			1
	3+ positive	1			
UPC	<0.5	68	54	9	46
	>0.5	4			
Microalbuminuria	Negative	51	46	8	33
	Low positive	7	5		5
	Medium positive	1			
	High positive	9	1		3
SDS-AGE ^a	Normal pattern	38	32	4	23
	Glomerular pattern	4			
	Mixed pattern	1		1	
	Tubular pattern		1		

^a SDS-AGE: Sodium dodecyl sulphate agarose gel electrophoresis.

there was a glomerular and tubular pattern at the same time. The urine test strip and the UPC were performed with fresh urine. Before the microalbuminuria test and the SDS-AGE were performed, the urine was frozen at -80°C .

Serological testing for *B. burgdorferi* has been described previously (Gerber et al., 2007). Dogs were considered to be positive for antibodies against *B. burgdorferi* if an enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against *B. burgdorferi* sensu lato was positive and the result could be confirmed by a Western blot examination. Data were recorded and analysed using a commercial computer programme (Statistical Package for the Social Sciences for Windows version 11, SPSS). Variables between BMDs and controls were compared using the Mann-Whitney *U*-test or Fisher's exact test. Differences were considered to be significant at $P < 0.05$.

Breed, age and gender of the dogs are presented in Table 1. A positive ELISA with a positive Western blot was present in 72/126 (57%) BMDs and 9/55 (16%) controls that met the inclusion criteria. This significant difference in seroprevalence ($P < 0.001$) is similar to the one of the population the dogs were selected from (58% and 15%, respectively) (Gerber et al., 2007). Three BMDs and six controls had been vaccinated against *B. burgdorferi*. Of these vaccinated dogs, two BMDs and two control dogs had antibodies against *B. burgdorferi*.

Results of protein measurements are shown in Table 2. There was no significant difference between BMDs and control dogs or between BMDs with antibodies against *B. burgdorferi* and those without in the occurrence of positive dipstick results, in the UPC value or the occurrence of UPC > 0.5 , in microalbuminuria results and in the occurrence of an abnormal urine protein pattern measured by SDS-AGE.

It was not possible to detect a higher prevalence of proteinuria as an early sign of renal disease in a large population of BMDs when compared to a population of dogs living in a similar environment or in BMDs with or without antibodies against *B. burgdorferi*. It is presumed that disease due to *B. burgdorferi* is rare compared to the exposure to the agent (Levy and Magnarelli, 1992). This is in agreement with the finding in healthy Labrador Retrievers, where no association between *B. burgdorferi* and microalbuminuria was found (Goldstein et al., 2007). However, considering the proposed pathophysiology of 'Lyme nephritis' as an immune mediated glomerular disease (Littman et al., 2006), a progressive course might be expected, with some dogs exhibiting early subclinical changes, such as proteinuria. Therefore it can be concluded that antibodies against *B. burgdorferi* are not associated with proteinuria as early sign of renal disease in BMDs.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

Acknowledgements

We thank the owners who volunteered to join the study after a call from the Swiss Club for Bernese Mountain dogs. This work was supported by the Swiss Club for Bernese Mountain Dogs, the Albert Heim Foundation and Novartis Animal Health Switzerland.

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Chapter 4

Follow- up of Bernese Mountain dogs and other dogs with serologically diagnosed *Borrelia burgdorferi* infection: What happens to seropositive animals?

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BioMed Central Veterinary Research 2009, 3:15

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Contributions of B. Gerber:

B. Gerber designed the study, analyzed the data and drafted the manuscript.

Research article

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Follow-up of Bernese Mountain dogs and other dogs with serologically diagnosed *Borrelia burgdorferi* infection: What happens to seropositive animals?

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Published: 8 May 2009

Received: 19 November 2008

BMC Veterinary Research 2009, 5:18 doi:10.1186/1746-6148-5-18

Accepted: 8 May 2009

This article is available from: <http://www.biomedcentral.com/1746-6148/5/18>

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Abstract

Background: Data on the long-term outcome of *B. burgdorferi* infections in adult dogs are sparse. The aim of the present study was to investigate whether Bernese Mountain dogs with serological evidence of natural *B. burgdorferi* infection more often develop signs such as lameness, azotemia or proteinuria during a follow-up period of 2.5 to 3.0 years. Seropositive Bernese Mountain dogs were compared to seronegative Bernese Mountain dogs and to seropositive and seronegative control dogs of other breeds.

Dogs included in a previous study on the prevalence of antibodies against *B. burgdorferi* in Bernese Mountain dogs were re-evaluated. Antibodies against *B. burgdorferi* were determined using an ELISA with a whole-cell sonicate as antigen and results were confirmed using a Western blot assay.

Results: Fifty-three Bernese Mountain dogs and 30 control dogs were re-evaluated. Re-evaluation was performed between 2.5 and 3.0 years (median 2.7 years) after the first assessment.

The age of the dogs at the second evaluation ranged from 3 to 11 years (median 6 years). There were no significant differences with regard to poor general condition or lameness between the first and the second evaluation.

At the first evaluation 22 (42%) of the Bernese Mountain dogs and 11 (37%) of the control dogs were considered positive for antibodies against *B. burgdorferi*. At the second evaluation 25 (47%) of the Bernese Mountain dogs and 12 (40%) of the control dogs were considered positive; 69% of the dogs showed the same serological result at both examinations and 31% were seroconverted or seroreverted. During the first examination, azotemia was diagnosed in 6 Bernese Mountain dogs and during the second examination in 11 Bernese Mountain dogs. No control dogs had azotemia in this study. In seropositive dogs there was no increase in lameness or signs of renal disease over time.

Conclusion: It may be concluded that antibodies against *B. burgdorferi* determined by whole cell ELISA and confirmed by Western blot were neither associated with the development of lameness nor with signs of renal disease like azotemia or proteinuria in dogs observed over a period of 2.5 to 3.0 years.

Background

Prior serologic surveys have shown that long-term persistence of *B. burgdorferi*-specific antibodies in dogs may occur. However, data on the long-term outcome of said infections in adult dogs are sparse [1,2]. In experimentally infected young beagles recurrent lameness was observed up to 280 days post infection [3]. In a post mortem study lymphadenopathy was found in the area of tick attachment and there were microscopical signs of inflammation in synovial membranes, joint capsules and tendon sheets after exposure to infected ticks [4]. Further observation lent some support to the assumption that canine *B. burgdorferi* infection is also related to renal disease [5,6]. The term "Lyme nephritis" was introduced to describe a renal disease with specific renal histopathologic lesions including immune-mediated glomerulonephritis, tubular necrosis and interstitial nephritis in dogs in which antibodies against *B. burgdorferi* were detected [6]. Considering the proposed pathophysiology of Lyme nephritis to be an immune-mediated glomerular disease [6,7] one would expect a progressive course with dogs developing disease after chronic infection. However, experimental infection of young Beagle dogs with *B. burgdorferi* sensu stricto gave no pathologic indications of glomerular disease for up to 581 days after experimental exposure to infected ticks [4]. It was speculated that under natural conditions age, breed or the pathological features of the borrelial agent might influence the development of Lyme nephritis. However, there are as yet no data on the long-term clinical outcome of natural *B. burgdorferi* infections in dogs. Recent studies partly support an interrelation between seroprevalence of *B. burgdorferi* and genetic predisposition for increased susceptibility to borrelial infections in Bernese Mountain dogs [8].

The aim of the present study was to investigate whether Bernese Mountain dogs with serological evidence of natural *B. burgdorferi* infection more often develop signs such as lameness, proteinuria or azotemia during a follow-up period of 2.5 to 3.0 years. Seropositive Bernese Mountain dogs were compared to seronegative Bernese Mountain dogs, and to seropositive and seronegative control dogs of other breeds.

Methods

Samples and dogs

Dogs included in a previous study on the prevalence of *B. burgdorferi* in Bernese Mountain dogs were re-evaluated after 2.5 years (899 days), and after 3.0 years (1113 days) (median 2.7 years (992 days)) [8]. This study focussed on including similar numbers of dogs of the following groups: Bernese Mountain dogs in which antibodies against *B. burgdorferi* were diagnosed, Bernese Mountain dogs in which no antibodies against *B. burgdorferi* were detected, control dogs (long haired large breed dogs but not Bernese Mountain dogs) in which antibodies against

B. burgdorferi were diagnosed, and control dogs in which no antibodies against *B. burgdorferi* were detected. For the first study dogs were sampled between July 2002 and April 2003. There were 160 Bernese Mountain dogs and 62 control dogs. Dogs were re-evaluated between June and October 2005. The owners of 82 Bernese Mountain dogs and 62 control dogs evaluated in the previous study were contacted a second time in order to evaluate the response over time of clinical and laboratory parameters. For 29 Bernese Mountain dogs (35%) and 32 control dogs (52%) no second examination was possible because the dogs were either dead (25 Bernese Mountain dogs and 14 control dogs) or the owners could not be reached a second time, or were unwilling to participate again (4 Bernese Mountain dogs and 18 control dogs). The health status of the dogs was assessed using a questionnaire filled in by the owners. Answers relating to general health and lameness were compared between the first and the second examination to assess possible consequences of a *B. burgdorferi* infection. Owners were asked to judge if the general health of their dog was normal or abnormal, and if their dogs were lame or not at the time of the second evaluation. At both time-points routine laboratory tests of blood and urine samples were performed. For serologic testing samples were frozen at minus 80°Celsius.

Haematology and serum biochemistry

Laboratory tests included a complete blood count (CBC) and a serum biochemical analysis containing determination of bilirubin, glucose, urea, creatinine, total protein, albumin, cholesterol, sodium, potassium, chloride, calcium and phosphorus concentrations; as well as the activity of alkaline phosphatase, alanine transferase, aspartate transferase and amylase. Hematocrit, urea, creatinine, total protein and albumin values during the first and the second examination were compared to assess renal function and function of the filtration barrier. Dogs were deemed azotemic if creatinine was above 125 µmol/L and/or if urea was above 9.4 mmol/L.

Urinalysis

Urinalysis consisted of a urine dip stick (Combur-Test, Roche Diagnostics GmbH, Mannheim Germany), microscopic examination of urine sediment, and determination of urine specific gravity. Results of urine protein measurements were considered only if fewer than 5 leukocytes per 400× field were counted in the urine sediment. The results of the protein measurement with the dip stick were recorded as negative or 1+ to 3+ positive. The urine protein-to-creatinine ratio (UPC) was measured on a Cobas-Integra analyzer (Roche, Rotkreuz, Switzerland). A difference in UPC between the first and the second examination was considered significant if it was 80% or more of the first value [9]. Microalbuminuria was measured by a commercial rapid immunoassay for canine microalbuminuria (E.R.D.-Screen™-Test, HESKA®, Fribourg, Switzerland).

Results were interpreted as negative, low-positive, medium-positive or high-positive according to the manufacturer's instructions. Urine specific gravity and results of the measurements of protein in urine were compared between the first and the second examination. If the dogs were azotemic, the azotemia was considered renal if the urine specific gravity was below 1.030.

Serologic testing

Serologic testing of serum stored at minus 80°C was performed. Serologic tests of the samples of the first and the second evaluation were performed together. An enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against *B. burgdorferi* sensu lato was performed for all dogs according to established protocols as described previously [8,10].

Briefly, a whole-cell sonicate of *B. burgdorferi* sensu stricto reference strain B31 (ATCC 35210), *B. garinii* N34, *B. afzelii* VS 461 and *B. valaisiana* VS 116 was used as antigen. Prior to serological testing serum samples were absorbed with a heterologous sorbant of washed formalin inactivated whole cells of *Escherichia coli*, *Salmonella typhimurium*, *Brachispira hyodysenteriae*, *Bacillus subtilis* and a total of ten serovars of *Leptospira* (*L.*) *interrogans* and *L. borgpetersenii*, respectively.

For each plate positive and negative control samples were applied. The cut-off was calculated from the serial measurement of the negative controls.

Western blot examinations to detect antibodies against *B. burgdorferi* were performed using a commercial test kit modified for dogs (Virion Ltd., Rueschlikon, Switzerland). Tests were performed according to the manufacturer's instructions and consisted of Western blot strips with defined partial antigens of *B. burgdorferi* sensu stricto and *B. afzelii*. Western blot results were interpreted according to criteria recommended for European species of *B. burgdorferi* sensu lato [11]. Samples were called positive if bands at the level of the partial antigens p100, p58, OspC, p21 or wb18 were identified or if at least two bands at the level of the partial antigens p45, bmpa und wb30 were present. Bands at the level of the partial antigens OspB, OspA, OspD, wb22 und OspE were considered unspecific.

Dogs were considered to have antibodies against *B. burgdorferi* if both the ELISA and the Western blot were positive.

Statistical analysis

Data was recorded and analyzed using a commercial computer program (Statistical Package for the Social Sciences for Windows version 11, SPSS Inc, Chicago Illinois, USA). Data of the first and the second examination was compared using the Wilcoxon signed rank test for ordinal data

and McNemar's change test for nominal data. Additionally, to compare age between dogs with and without poor general condition and between dogs with and without lameness the Mann-Whitney U test was used. For dogs re-evaluated after the first examination and those not re-evaluated the Mann-Whitney U test was also used for the comparison of hematocrit, urea, creatinine, protein, albumin, urine specific gravity and urine protein-to creatinine ratio. The Fisher's exact test was used to compare the occurrence of lameness between dogs re-evaluated a second time and those not re-evaluated. Differences were considered significant at $P < 0.05$. Bonferroni correction was applied in the comparison of haematology, serum chemistry and urine parameters.

Results

Fifty-three Bernese Mountain dogs and 30 control dogs were re-evaluated. The age of the dogs at the time of the second evaluation ranged from 3 to 11 years (median 6 years). There were 23 male and 60 female dogs.

Antibodies against *B. burgdorferi*

At the first evaluation, 22 (42%) of the Bernese Mountain dogs and 11 (37%) of the control dogs were considered positive for antibodies against *B. burgdorferi* (Table 1). At the second evaluation, 25 (47%) of the Bernese Mountain dogs and 12 (40%) of the control dogs were considered positive.

Of the Bernese Mountain dogs, 36 (68%) had the same serological results at both examinations and 17 (32%) either seroconverted or seroreverted. Of the control dogs, 21 (70%) had the same serological result at both examinations and 9 (30%) either seroconverted or seroreverted. There was no significant change in serological results between the first and the second evaluation in either group.

Answers to questionnaire

All dogs were considered healthy by their owners at the time of the first evaluation. At the second evaluation, 8 owners stated that the general condition of their dogs had deteriorated (Table 2). Of these dogs 1 Bernese Mountain dog and 2 control dogs were seropositive for *B. burgdorferi* in both examinations, 2 control dogs were negative in both examinations, 1 Bernese Mountain dog seroconverted and 1 Bernese Mountain dog and 1 control dog seroreverted. Three of the eight dogs also showed lameness at the time of the second evaluation. (2 seroreverted and 1 remained seropositive). There were no significant differences in the occurrence of a poor general condition between the first and the second evaluation. However, dogs with a poor general condition were significantly older than those with a good general condition at the time of the second evaluation (age 8 to 11 years, median 10 years versus 3 to 10 years, median 6 years; $P < 0.001$).

Table 1: Antibodies against *B. burgdorferi* in Bernese Mountain dogs and control dogs at the first evaluation and after a median of 2.7 years.

Serology result at the second evaluation	First evaluation			
	Bernese Mountain dogs (N = 53)		Control dogs (N = 30)	
	<i>B. burgdorferi</i> serology			
	positive (N = 22)	negative (N = 31)	positive (N = 11)	negative (N = 19)
remained negative		21 (68%)		14 (74%)
remained positive	15 (68%)		7 (64%)	
seroconverted*		7 (32%)		4 (36%)
seroreverted**	10 (32%)		5 (26%)	

*seroconversion: Serologic test for antibodies against *B. burgdorferi* was negative in the first evaluation and changed to positive in the second.

**seroreversion serologic test for antibodies against *B. burgdorferi* was positive in the first evaluation and changed to negative in the second.

Five owners reported lameness in their dogs at the first examination and 10 owners reported lameness at the second evaluation (Table 1). Of the 10 dogs lame at the second evaluation, poor general condition was reported for 3 (2 seroreverted and 1 remained seropositive). Four of the dogs that were lame at the first evaluation were still lame at the second evaluation, whilst 1 seronegative Bernese Mountain dog was no longer lame. Six dogs were newly lame at the second evaluation (5 Bernese Mountain dogs and 1 control dog). Five of these dogs showed antibodies

against *B. burgdorferi* at the first evaluation whilst 1 Bernese Mountain dog proved negative. However, 2 Bernese Mountain dogs seroreverted and were negative at the second evaluation, whilst the other dogs kept their serological status. There was no significant difference in the occurrence of lameness between the first and the second evaluation in all groups. Dogs with lameness at the second evaluation were significantly older than the dogs that showed no lameness (6 to 11 years, median 8 years versus 3 to 10 years, median 6 years; $P < 0.005$).

Table 2: General condition and lameness in Bernese Mountain dogs and control dogs at the first examination and after a median of 2.7 years.

			Bernese Mountain dogs (N = 53)	Control dogs (N = 30)		
			Result of <i>B. burgdorferi</i> serology at the first examination			
			positive (N = 22)	negative (N = 31)	positive (N = 11)	negative (N = 19)
General condition	1 st ex.	normal	21	31	11	18
		poor				
	2 nd ex.	normal	19	30	8	16
		poor	2	1	3	2
		[P = 0.5]*	[P = 1.0]	[P = 0.25]	[P = 0.5]	
Lameness	1 st ex.	no	21	30	10	15
		yes		1	1	3
	2 nd ex.	no	17	30	9	15
		yes	4	1	2	3
		[P = 0.125]	[P = 1.0]	[P = 1.0]	[P = 1.0]	

ex. = examination

*P resulting from comparison between first and second evaluation

Results of laboratory analysis

Results of the laboratory tests and urinalysis are shown in Table 3. At the first examination, 6 Bernese Mountain dogs were azotemic (creatinine 95–155 $\mu\text{mol/L}$, median 109 $\mu\text{mol/L}$; urea 7.1–13.1 mmol/L, median 10.1 mmol/L). Renal azotemia was considered a possibility in 5 of these dogs. At the second examination 11 Bernese Mountain dogs were azotemic (creatinine 82–260 $\mu\text{mol/L}$, median 130 $\mu\text{mol/L}$; urea 6.5–15.1 mmol/L, median 10.0 mmol/L). In 5 of these dogs renal azotemia was considered possible. No control dogs were azotemic at either

examination. Of the 6 Bernese Mountain dogs which were azotemic at the first examination 2 were non azotemic at the second evaluation whilst 4 remained azotemic. At the first examination all azotemic dogs were negative for antibodies against *B. burgdorferi*. At the second examination, 6 of the 11 dogs with azotemia were positive for antibodies against *B. burgdorferi*.

Six Bernese Mountain dogs and 3 control dogs had a significant increase in UPC between the first and the second examination (80% or more of the first value; 0.06–0.44,

Table 3: Range and median of hematology, serum chemistry and urine parameters in Bernese Mountain dogs and control dogs at the first examination and after a median of 2.7 years.

			Bernese Mountain dogs (N = 53)	Control dogs (N = 30)		
			Result of <i>B. burgdorferi</i> serology at the first examination			
Parameters	Reference range		positive (N = 22)	negative (N = 31)	positive (N = 11)	negative (N = 19)
Hematocrit [%]	42–55	1 st ex.	41–63 (51)	41–60 (50)	40–57 (51)	43–54 (48)
		2 nd ex.	36–54 (46)	36–54 (46)	34–49 (44)	41–55 (47)
			[P < 0.001]*	[P < 0.001]*	[P = 0.004]*	[P = 0.06]
Urea [mmol/L]	3.8–9.4	1 st ex.	3.1–8.9 (6.7)	4.0–13.1 (6.5)	3.8–7.7 (5.6)	3.4–8.6 (5.6)
		2 nd ex.	3.9–13.7 (6.5)	3.6–15.1 (6.9)	2.8–6.4 (4.8)	1.7–6.7 (4.2)
			[P = 0.73]	[P = 0.34]	[P = 0.21]	[P = 0.001]*
Creatinine [μmol/L]	64–125	1 st ex.	80–120 (102)	74–155 (100)	72–119 (87)	66–114 (82)
		2 nd ex.	64–138 (108)	65–260 (99)	59–110 (78)	52–107 (72)
			[P = 0.85]	[P = 0.96]	[P = 0.003]*	[P < 0.001]*
Protein [g/L]	56–71	1 st ex.	58–74 (64)	43–74 (63)	53–65 (61)	50–67 (61)
		2 nd ex.	53–70 (64)	55–73 (64)	59–69 (61)	58–74 (63)
			[P = 0.77]	[P = 0.002]*	[P = 0.28]	[P = 0.02]
Albumin [g/L]	29–37	1 st ex.	25–33 (30)	21–34 (30)	28–35 (32)	28–35 (31)
		2 nd ex.	22–36 (33)	28–38 (35)	30–40 (33)	30–40 (35)
			[P = 0.001]*	[P < 0.001]*	[P = 0.03]	[P < 0.001]*
Urine specific gravity		1 st ex.	1.010–1.049 (1.030)	1.006–1.050 (1.028)	1.011–1.048 (1.021)	1.007–1.048 (1.027)
		2 nd ex.	1.006–1.050 (1.030)	1.007–1.050 (1.034)	1.012–1.046 (1.023)	1.006–1.042 (1.026)
			[P = 0.21]	[P = 0.27]	[P = 0.81]	[P = 0.41]
Urine protein-to-creatinine ratio (UPC)		1 st ex.	0.06–0.28 (0.11)	0.06–0.42 (0.11)	0.07–0.48 (0.12)	0.08–0.21 (0.11)
		2 nd ex.	0.05–0.36 (0.14)	0.06–0.44 (0.10)	0.06–0.92 (0.09)	0.06–0.53 (0.11)
			[P = 0.05]	[P = 0.79]	[P = 0.13]	[P = 0.91]
Urine protein on dip stick		1 st ex.	15 neg., 3 pos.	21 neg., 5 pos.	9 neg., 2 pos.	11 neg., 6 pos
		2 nd ex.	6 neg., 11 pos.	7 neg., 18 pos.	4 neg., 7 pos.	9 neg., 8 pos.
			[P = 0.001]*	[P < 0.001]*	[P = 0.03]	[P = 0.27]
Micro-albuminuria test		1 st ex.	11 neg., 8 pos.	22 neg., 4 pos.	10 neg.	14 neg., 3 pos.
		2 nd ex.	11 neg., 6 pos.	20 neg., 5 pos.	10 neg., 1 pos.	13 neg., 4 pos.
			[P = 0.69]	[P = 0.25]	[P = 1.00]	[P = 1.00]

*Significant difference between first and second examination at $P < 0.006$ due to Bonferroni correction.
ex. = examination

median 0.20). Four of the Bernese Mountain dogs and 1 of the control dogs had antibodies against *B. burgdorferi* at the first examination. At the second evaluation one more Bernese Mountain dog was positive. All other dogs kept their serologic status.

Of the 16 dogs with a positive dipstick result in the first examination, 4 were negative in the second examination (1 Bernese Mountain dog and 3 control dogs). A total of 21 Bernese Mountain dogs and 11 control dogs changed from a negative dipstick result in the first examination to a positive result in the second examination (30 dogs 1+ positive and 2+ positive). In 7 of the 32 dogs with a change in the dipstick result positive antibodies against *B. burgdorferi* were detected in the first examination and these dogs remained positive, 13 were negative and remained negative, 6 seroconverted and 6 seroreverted. The difference in occurrence of a positive dipstick result between the first and the second examination was significant in Bernese Mountain dogs but not in control dogs.

Overall, 15 (18%) of the dogs were tested positive against microalbuminuria during the first examination and 16 (19%) during the second test. Three dogs were low-positive in the first examination and tested negative in the second examination. Four dogs were high-positive in both the first and second examination. Seven dogs that were negative during the first examination were positive during the second examination (5 light positive and 2 medium positive). Of the 7 dogs in which the microalbuminuria test changed from negative to positive, 6 were Bernese Mountain dogs, and 1 was a control dog. In 3 of these 7 dogs antibodies against *B. burgdorferi* were detected during the first examination and remained positive, 3 were negative and remained negative and 1 seroconverted.

Comparison with dogs not evaluated a second time

Not all dogs evaluated in a previous study [8] were examined a second time. To confirm that a representative number of the dogs evaluated the first time was selected, different parameters of the first examination were compared between these dogs and those that were not re-examined. The only significant difference was found in the hematocrit of seronegative control dogs. The hematocrit was higher in dogs evaluated a second time ($P = 0.001$), ranging from 43–54% (median 48%) in re-evaluated dogs and from 35–53% (median 44%) in non re-evaluated dogs. Seven of the dogs that were not re-evaluated had a hematocrit below the reference range (42–55%).

Discussion

The purpose of this study was to re-evaluate similar numbers of Bernese Mountain dogs and control dogs in which antibodies against *B. burgdorferi* had been detected or not

detected in a previous study [8]. However, even though all owners of control dogs participating in the first study were re-contacted, we were not able to obtain as high a number of control dogs positive for antibodies against *B. burgdorferi* as Bernese Mountain dogs. The main reason was that owners of control dogs were not willing to participate in the study a second time.

We were not able to obtain sufficient useful information on relevant diseases or treatments of the dogs between the two examinations. Accordingly, the influence of past disease problems on the current status of the dogs could not be evaluated. However, comparing the dogs which were re-evaluated with those that were not re-evaluated indicated that a representative group of dogs from the first study was included.

In dogs that were re-evaluated there were only minor differences between the first and the second examination. For some dogs owners reported a poorer general condition at the second evaluation. The unspecific parameter might indicate non obvious health problems. This was considered important because in human medicine non-specific signs like persistent pain and fatigue are attributed to "chronic Lyme disease", which is unfortunately not a well defined term [12]. However, in most dogs poor general condition could be attributed to age-related changes.

Lameness and fever were the only clinical findings in experimentally infected dogs [13]. For this study it was assumed that lameness would be more common in dogs that were positive for *B. burgdorferi* antibodies than in other dogs. However, this was not the case. There are several explanations for this. Possibly *B. burgdorferi* did not cause disease in these dogs at all, or the disease remained subclinical. In addition, it is known that clinical signs are often intermittent and lameness was assessed only at the time-points of both examinations. The question also remains whether European *B. burgdorferi* strains might cause different or no diseases in dogs. The reports on Lyme disease in dogs originate from the United States, where only *B. burgdorferi* sensu stricto are present whilst in Switzerland *B. garinii* predominates, followed by *B. afzelii* and *B. burgdorferi* sensu stricto [14,15]. Furthermore, even within the species of *B. burgdorferi* sensu stricto different genotypes were shown to cause different disease severities in mice [16]. Still more dogs which were seropositive at the first evaluation were lame at the second evaluation; however, if *B. burgdorferi* was involved in the development of lameness one would expect persistent infection and not a seroreversion. Furthermore, lameness due to other arthropathies is likely in old large breed dogs. Therefore, we come to the same conclusion as Levy and Magnarelli (1992)[17], i.e. that the serologic status of apparently healthy dogs has no value in predicting subsequent lameness.

Seroconversion and seroreversion were similar in both examinations, indicating that the percentage of dogs infected might remain stable over time. This differs from a study in an endemic area, where more dogs seroconverted (24%) than seroreverted (9%) [17]. Different strains or recurrent infection might be the reason for this. Interestingly, in humans exposed to ticks seroreversion seems to be more common than seroconversion [18]. Seropositivity is also influenced by the season in which the samples are collected. OD values were found to be lower during the ticks' quiescent period than during the tick season [19]. For the present study the first sampling was partly conducted during the non-tick season. This might have led to fewer positive samples than during the tick-season.

There was no indication of reduced renal function in the parameters examined. Although serum creatinine was higher in the dogs with antibodies against *B. burgdorferi*, azotemia rarely occurred. Furthermore, there were no differences in serum urea concentrations.

The serum albumin concentration was lower in Bernese Mountain dogs than in control dogs. This could not be attributed to urinary loss since the degree of proteinuria in Bernese Mountain dogs was not different from that in other dogs. This corresponds to earlier studies in which no higher prevalence of proteinuria was found in Bernese Mountain dogs or Labrador Retrievers with antibodies against *B. burgdorferi* [20,21]. The only significant difference with regard to protein excretion in the urine was an increase in positive dipstick results in Bernese Mountain dogs during the second examination. As this was the case in both positive and negative dogs an influence of *B. burgdorferi* infection is unlikely. The slightly higher specific gravity in the group of negative Bernese Mountain dogs might explain some of the changes. Furthermore, most dipstick readings changed from negative to only 1+ positive, a change that is minimal and might even depend on the reader.

The test used to establish microalbuminuria had predicted the development of proteinuria in dogs with X-linked hereditary glomerulopathy [22]. This study did not reveal a difference in microalbuminuria between the first and the second examination. The occurrence of microalbuminuria was similar to another report in which 19% of the healthy dogs showed microalbuminuria [23]. The prevalence of microalbuminuria has been reported to increase with age, but in this study there were minimal increases between the first and the second evaluation even though the dogs were almost three years older [24].

Conclusion

It may be concluded that antibodies against *B. burgdorferi* determined by whole cell ELISA and confirmed by West-

ern blot were not associated with the development of lameness and signs of renal disease like azotemia or proteinuria in dogs observed over a period of 2.5 to 3.0 years.

Authors' contributions

BG: Designed the study, analyzed the data and drafted the manuscript

KH: Contributed to the study design, collected the data for the second evaluation and contributed to the manuscript drafting and data interpretation.

SE: Collected and interpreted the data for the first evaluation and contributed to the study design.

CER: Was involved in the study design and coordination and contributed to the critical evaluation and interpretation of the data.

MMW: Performed the serologic tests, was involved in the study design and the drafting of the manuscript.

Acknowledgements

We thank the owners who volunteered to join the study following a call from the Swiss Club for Bernese Mountain dogs. This work was supported by the Swiss Club for Bernese Mountain dogs and the Albert Heim Foundation.

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Chapter 5

Complement C3 in Bernese Mountain dogs

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Veterinary Clinical Pathology 2010; 39(2); 164-168

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B. Gerber designed the study, analyzed the data and drafted the manuscript.

ORIGINAL RESEARCH

Complement C3 in Bernese Mountain dogs

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Key Words

Bernese Mountain dog, *Borrelia burgdorferi*, complement C3, glomerulonephritis

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DOI:10.1111/j.1939-165X.2009.00205.x

Background: Previous research suggests that low serum concentrations of the third component of complement (C3) are associated with both the susceptibility to infectious agents such as *Borrelia burgdorferi* and the development of glomerular disease. We hypothesized that low levels of C3 are associated with the coincident occurrence of *B. burgdorferi* infection and glomerulonephritis in Bernese Mountain dogs.

Objectives: The aims of this study were to evaluate the serum concentration of C3 in Bernese Mountain dogs with and without antibodies against *B. burgdorferi* and to compare this concentration with that of healthy control dogs.

Methods: Eighty-three clinically healthy Bernese Mountain dogs and 46 control dogs were included. Antibodies against *B. burgdorferi* were determined using an ELISA with a whole cell sonicate as antigen. Results were confirmed using Western blot. C3 was measured using a single radial immunodiffusion test. Results were reported as the percentage concentration of C3 compared with that in pooled preserved canine serum (100% C3 concentration).

Results: Median C3 concentration was 128.5% in Bernese Mountain dogs with antibodies against *B. burgdorferi*, 133.5% in *B. burgdorferi*-negative Bernese Mountain dogs, 87.8% in positive control dogs, and 102.2% in negative control dogs. Within Bernese Mountain and control groups, C3 was lower in dogs with antibodies against *B. burgdorferi* compared with those without. Percentage concentration of C3 was higher in healthy Bernese Mountain dogs compared with control dogs.

Conclusion: Low C3 concentration is not an explanation for the high prevalence of *B. burgdorferi* infections and glomerular disease in Bernese Mountain dogs.

Introduction

The complement system is a central part of innate immunity and is important for defense against infectious agents including *Borrelia burgdorferi* sensu lato.¹ Innate and acquired immunity together are important for the elimination of *B. burgdorferi*. In the presence of antibodies, complement-mediated killing of *B. burgdorferi* is responsible for eradication of the bacteria. Yet some strains of *B. burgdorferi* can evade complement attack, possibly by binding complement-inhibiting factors H and factor H-like protein 1 in serum.² Other strains of *Borrelia* are unable to bind these factors and

therefore are susceptible to complement attack. This applies to strains of *Borrelia garinii*, the most common genospecies in Switzerland, and some *B. burgdorferi* sensu stricto strains.^{2,3}

Animal models of immune-complex-mediated renal disease have demonstrated a protective role for complement.⁴ Membranoproliferative glomerulonephritis in humans is often seen in conjunction with an antibody directed against the third component of complement (C3) convertase. This antibody (C3-nephritic factor) prolongs the half-life of C3 convertase, which leads to C3 depletion. C3-nephritic factor was found in 88% of children with membranoproliferative

glomerulonephritis Type II, and in most children, the C3 level concentration in serum was low at some point during the disease.⁵ In addition it has been shown that C3 deficiency was associated with glomerular disease.⁶ Dogs with genetically determined C3 deficiency demonstrated increased susceptibility to renal disease and infections.⁷ Five of 20 dogs with a complete deficiency of C3 developed clinical evidence of renal disease, while 14 of 15 remaining dogs had histologic evidence of membranoproliferative glomerulonephritis. These findings indicate that low levels of C3 are associated with both the susceptibility to infectious agents such as *B. burgdorferi* and the development of glomerular disease.

Both infection with *B. burgdorferi* and glomerulonephritis are common in Bernese Mountain dogs.^{8,9} Bernese Mountain dogs tend to develop glomerulonephropathy that in most cases has been recognized as membranoproliferative glomerulonephritis.^{9,10} It also has been found that the proportion of Bernese Mountain dogs with antibodies against *B. burgdorferi* was considerably higher than that in dogs of other breeds from the same environment.⁸ One might, therefore, ask whether *B. burgdorferi* infection and glomerular disease in this breed are linked. A relationship between renal disease and infection with *B. burgdorferi* in dogs was suspected in earlier reports. However, a causative role of *B. burgdorferi* in the development of renal disease was not confirmed,^{9,11–13} and renal lesions were not found in experimentally infected dogs.¹⁴

We hypothesized that in Bernese Mountain dogs, low levels of complement C3 are associated with the coincident occurrence of *B. burgdorferi* infection and glomerulonephritis. The aims of this study were to evaluate the serum concentration of C3 in Bernese Mountain dogs with and without antibodies against *B. burgdorferi* and to compare this with the C3 concentration in healthy control dogs.

Material and Methods

Dogs and samples

Samples were collected between July 2002 and April 2003 from 2 groups of dogs: clinically healthy Bernese Mountain dogs and healthy long-haired, large-breed control dogs. The latter were selected to resemble Bernese Mountain dogs in size and hair coat. All of the dogs were part of a previous study of the prevalence of *B. burgdorferi* infection in Bernese Mountain dogs.⁸ Health status of the dogs was assessed by a questionnaire filled out by the owners. Furthermore, CBCs, serum chemistry analysis, and urinalysis

were performed. Dogs were excluded if they had neutrophilia (neutrophils > 7500/ μ L), azotemia (urea > 9.4 mmol/L and/or creatinine > 125 μ mol/L), had > 4 WBCs/ \times 40 field in urine sediment preparations, if the urine protein:creatinine ratio was > 0.5, or if urine bacterial culture was positive.

Serologic testing

An ELISA for the detection of antibodies against *B. burgdorferi* sensu lato was performed in all dogs according to a method described previously.¹⁵ Briefly, a whole cell sonicate of *B. burgdorferi* sensu stricto reference strain B31 (ATCC 35210) was used as antigen. The samples were previously absorbed with a heterologous sorbant consisting of washed formalin-inactivated whole cells of *Escherichia coli*, *Salmonella typhimurium*, *Brachispira hyodysenteriae*, *Bacillus subtilis*, and leptospires comprising 18 serovars.

Western blot examination for the detection of antibodies against *B. burgdorferi* was performed in all but 1 dog with a negative ELISA result. For the study, a commercial test kit (*B. burgdorferi* IgG Western Blot, Virion Ltd., Rueschlikon, Switzerland) was adapted for use in dogs. The tests were performed according to the manufacturer's instructions and consisted of Western blot strips with defined partial antigens of *B. burgdorferi* sensu stricto and *B. afzelii*. In preliminary tests with positive and negative canine serum, an antigen dilution of 1:200 and a conjugate (alkaline-phosphatase rabbit anti-canine IgG, H+L, Sigma, Diesenhofen, Switzerland) dilution of 1:2000 were considered adequate. Western blot results were interpreted according to criteria recommended for 3 European species of *B. burgdorferi* sensu lato.¹⁶ Samples were considered positive if bands at the level of partial antigens p100, p58, OspC, p21, or wb18 were identified or if at least 2 bands at the level of partial antigens p45, bmpa, and wb30 were present. Bands at the level of partial antigens OspB, OspA, OspD, wb22, and OspE were considered too nonspecific (negative). With human samples, these criteria for interpretation of Western blot results had a sensitivity of 50.8–56.1% and a specificity of 96.5–97.9%, depending on the *B. burgdorferi* species.¹⁶

Complement measurement

Serum was stored at -80°C for up to 12 months from the time of collection before testing. Complement factor C3 was determined by single radial immunodiffusion. Agar was produced by placing 5 mL of 1% agarose (Amersham Bioscience Europe GmbH, Freiburg, Germany) and 2 mL of goat anti-canine C3

(Bethyl Laboratories, Montgomery, TX, USA) in a Petri dish at 56°C. Serum (15 µL) diluted 1:4 (to conserve samples) was added into punched wells and incubated for 72 hours in a humid chamber. The test was evaluated using commercial complement in pooled preserved canine serum (Innovative Research, Novi, MI, USA). Within-day coefficients of variation (CV) for precision in a series of 10 repeated measurements of 5 samples ranged from 0.82% to 1.83%. Between-day CVs measured in 5 samples on each of 10 consecutive days ranged from 0.92% to 1.82%. Complement in the commercial pooled canine serum was considered as 100% concentration and was used to establish a dilution curve, which was exponential. The amount of C3 in test samples was expressed as a percentage of the pooled serum.

Statistical analysis

Data were recorded and analyzed using a commercial computer program (Statistical Package for the Social Sciences for Windows 15, SPSS Inc., Chicago, IL, USA). According to the results of the test for antibodies against *B. burgdorferi*, dogs were divided into subgroups: subgroup A, Bernese Mountain dogs with antibodies against *B. burgdorferi*; subgroup B, Bernese Mountain dogs without antibodies; subgroup C, control dogs with antibodies; and subgroup D, control dogs without antibodies. Between and within Bernese Mountain dogs and control dogs variables were compared using the Mann-Whitney *U*-test for the evaluation of age and C3 percentage concentration. C3 percentages were compared between groups, between subgroups within groups, and between subgroups A and B and subgroup D. Fisher's exact test was used for the comparison of gender and seroprevalence of *B. burgdorferi* antibodies. Differences were considered significant at $P < .05$.

Results

Eighty-three healthy Bernese Mountain dogs and 46 control dogs were included in the study. Breed, age, and gender of the dogs are shown in Table 1. Bernese Mountain dogs were significantly younger than control dogs ($P = .020$). Gender distribution was not significantly different between the 2 groups. Seroprevalence of *B. burgdorferi* in Bernese Mountain dogs (61%) was significantly different than in control dogs (15%) ($P < .001$).

The percentage concentration of C3 was 78.3–174.4% (median 128.5%) in subgroup A, 110.3–211.0% (133.5%) in subgroup B, 55.5–114.6%

Table 1. Breed, gender, and age of dogs included in the study.

Breed	No. of Dogs			Age (years)	
	Total	Female	Male	Range	Median
Bernese Mountain dogs	83	58	25	1–11	4*
Control dogs	46	28	18	1–11	5
Landseer	24	14	10	1–11	5
Newfoundland	7	6	1	4–8	7
Flat-Coated Retriever	7	4	3	1–7	1
Golden Retriever	2	2	0	1–5	3
Belgian Shepherd	1	1	0	2	2
Tibetan Mastiff	1	0	1	5	5
Mixed breed	4	1	3	4–8	7

*Significantly different from control dogs ($P = .020$, Mann-Whitney *U*-test).

(87.8%) in subgroup C, and 55.5–114.6% (87.8%) in subgroup D (Figure 1). The percentage concentration of C3 was significantly lower in Bernese Mountain dogs with antibodies against *B. burgdorferi* compared with those without ($P = .028$) (Figure 1). The percentage concentration of C3 was significantly lower in dogs in subgroup D compared with dogs in subgroups A ($P = .001$) and B ($P < .001$). The percentage concentration of C3 in all healthy Bernese Mountain dogs was significantly higher compared with healthy control dogs ($P < .001$).

One Bernese Mountain dog with antibodies against *B. burgdorferi* (subgroup A; C3 = 123.7%), 2

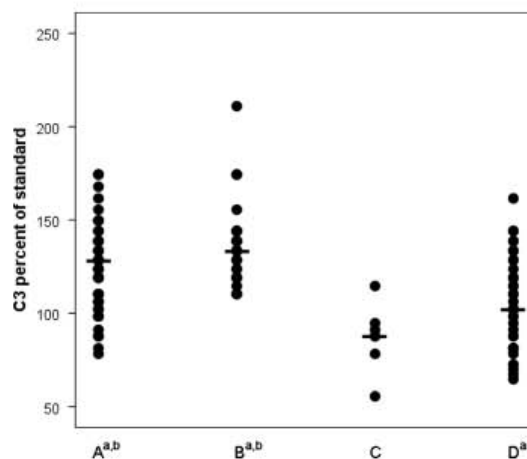


Figure 1. Percentage concentration of third component of complement (C3) in Bernese Mountain Dogs and control dogs. (A) Bernese Mountain Dogs with antibodies against *Borrelia burgdorferi*, $n = 51$; (B) Bernese Mountain Dogs without antibodies, $n = 32$; (C) control dogs with antibodies, $n = 7$; (D) control dogs without antibodies, $n = 39$. Dots are individual animals and indicate range; bar indicates median. Groups with the same superscript letter are significantly different. No statistical analysis was performed between groups C and A or B.

seropositive control dogs (subgroup C; C3 = 87.8% and 91.2%), and 4 seronegative control dogs (subgroup D; C3 = 94.7–133.5%) had been vaccinated against Lyme borreliosis.

Discussion

In healthy Bernese Mountain dogs, percentage concentrations of C3 in serum were not low. This result does not support the hypothesis that low C3 concentration increases the susceptibility to *B. burgdorferi* infection or the development of glomerular disease in Bernese Mountain dogs. Unexpectedly, we determined that the percentage concentration of C3 was higher in healthy Bernese Mountain dogs compared with healthy control dogs. The reason for the higher level of C3 is not clear. Because Bernese Mountain dogs are susceptible to histiocytic sarcomas, it could be speculated that active dendritic cells contribute to increased serum concentrations of C3. Histiocytic sarcomas likely arise from dendritic cells, which have been shown to produce complement C3 in humans and mice.^{17,18} Still, within the group of healthy Bernese Mountain dogs, C3 levels were lower in dogs with antibodies against *B. burgdorferi* compared with those without. Even though this would support the hypothesis of this study, it was not considered relevant because there was marked overlap in C3 levels in these 2 subgroups and the percentage concentration of C3 in positive Bernese Mountain dogs was only slightly lower compared with negative Bernese Mountain dogs. In contrast, dogs with genetically determined C3 deficiency have levels of C3 as low as 3.6–11.1% of the mean of normal dogs.¹⁹ Thus, the slightly lower C3 concentration in healthy Bernese Mountain dogs with antibodies against *B. burgdorferi* might be the result of another mechanism rather than an inborn error of the breed. One mechanism might be immune complex disease, like such as occurs with infection with *B. burgdorferi*.²⁰

Low C3 concentration is described in different forms of primary glomerular disease in humans, for example, membranoproliferative glomerulonephritis or acute poststreptococcal glomerulonephritis.²¹ Low serum C3 concentrations were not found in a recent study of dogs with protein-losing nephropathy.²² Because C3 is an acute phase protein, one explanation for a normal C3 concentration despite underlying C3 deficiency is an acute phase reaction that increases C3 levels in patients with systemic infection or chronic inflammation.^{20,23–25}

In the present study, antibodies against *B. burgdorferi* were measured using a widely accepted 2-step

procedure using a whole cell ELISA followed by a Western blot to confirm the results.²⁶ With the Western blot, false-positive ELISA results should be eliminated, including false-positive results from vaccinated dogs. This test procedure was found to be in moderate agreement with an ELISA to measure C6 protein in dogs in Europe (agreement rate 79%, κ 0.571).²⁷ However, antibodies against *B. burgdorferi* do not prove active infection, as antibodies can persist for years even if the animal has recovered from the infection.²⁶ Still, persistent antibodies were unlikely to have interfered with the results of this study as serum levels of C3 were higher in all Bernese Mountain dogs compared with control dogs, independent of *B. burgdorferi* status. Only a few dogs in this study were vaccinated, and vaccination did not seem to influence the results.

In conclusion, low percentage concentrations of C3 are not an explanation for the high prevalence of *B. burgdorferi* infections and glomerular disease in Bernese Mountain dogs.

Disclosure: The authors have indicated they have no affiliations or financial involvement with any organization or entity with a financial interest in, or in financial competition with, the subject matter or materials discussed in this paper.

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Chapter 6

Comparison of a rapid immunoassay for antibodies to the C6 antigen with conventional tests for antibodies against *B. burgdorferi* in dogs in Europe

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The Veterinary Record 2009; 165(20); 594-597

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B. Gerber designed the study, analyzed the data and drafted the manuscript.

Comparison of a rapid immunoassay for antibodies to the C6 antigen with conventional tests for antibodies to *Borrelia burgdorferi* in dogs in Europe

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A commercial immunoassay for antibodies to the C6 antigen of *Borrelia burgdorferi* was evaluated against an IgG in-house ELISA in combination with a Western blot assay to examine 104 samples of serum from 53 healthy Bernese mountain dogs, which were suspected to have a breed predisposition to Lyme borreliosis, and 55 samples from 30 healthy large-breed longhair dogs. The two test methods correlated in 125 (79 per cent) of the samples with an agreement of kappa=0.571 ($P<0.001$). In comparison with the in-house ELISA in combination with a Western blot, the sensitivity and specificity of the C6 test were 81 per cent and 77 per cent respectively. The agreement between the tests was better with the samples from the Bernese mountain dogs ($k=0.681$) than with the samples from the control dogs ($k=0.347$).

THE diagnosis of Lyme borreliosis in dogs can be difficult owing to its variable clinical signs (Littman and others 2006). Erythema migrans is a distinct sign of the infection in human beings, but does not occur in dogs (Stanek and Strle 2003, Greene and Straubinger 2006). The lack of accuracy and standardisation of the serological tests increases

the difficulty (Jacobson and others 1996). However, tests for antibodies to *B. burgdorferi* sensu lato (further referred to as *B. burgdorferi*) are still used because it is not easy to detect the organism. ELISA and indirect fluorescent antibody tests, in combination with a Western blot assay, are widely used for the diagnosis of Lyme borreliosis (Littman and others 2006), even though differences between them and their interpretation can lead to conflicting results (Jacobson and others 1996). The problem is complicated by the fact that different species are responsible for the disease in the USA and Europe. In the USA *B. burgdorferi* sensu stricto is the only agent causing the illness in people, whereas in Europe it is mostly caused by *B. afzelii* and *B. garinii* (Steere 2001). In dogs the role of *B. burgdorferi* in clinical illness is less clear, but they are considered a sentinel animal for Lyme disease in people and might provide a convenient measure of the potential risk to people of *B. burgdorferi* (Lindenmayer and others 1991, Goossens and others 2001). It would therefore be valuable to have an accurate and standardised test for the serodiagnosis of borreliosis in dogs.

The recombinant peptide C6 is used as the antigen in more recent ELISAs. It corresponds to the immunodominant region (IR6) of the VlsE surface protein (Vmp-like sequence, expressed) and was originally isolated from a European species of *B. garinii* (Greene and Straubinger 2006). This region is genetically, structurally and antigenically conserved in *B. garinii*, *B. afzelii* and other strains of *B. burgdorferi*. The genes for IR6 are only expressed during replication in the mammalian host; the protein has not been found in ticks that had not fed on the host or in ticks kept under temperature conditions used for vaccines or diagnostic antigens (Greene and Straubinger 2006). The peptide is considered to be a marker for active infection and is increasingly replacing the conventional methods with whole-cell ELISAs in combination with an immunoblot (Marques and others 2002, Bacon and others 2003). In human beings C6 ELISAs are 99 per cent specific for the diagnosis of borreliosis, and their sensitivity varies between 62 per cent and 100 per cent depending on the phase of the disease (Liang and others 1999). It has been reported that European patients show a restricted antibody response, possibly leading to a lower sensitivity of serodiagnosis (Dressler and others 1994, Hauser and others 1997), but the C6 ELISA has proved reliable in human patients from Europe with clinically well defined Lyme disease (Liang and others 2000a).

In studies with dogs infected experimentally with *B. burgdorferi*, antibodies to C6 increased more rapidly after infection and decreased more rapidly after treatment than antibodies measured with a whole-cell ELISA (Liang and others 2000b, Straubinger and others 2000, Philipp and others 2001). A further advantage of the C6 ELISA is that it does

Veterinary Record (2009) 165, 594-597

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the **VETERINARY RECORD** | November 14, 2009

not cross-react with infections such as *Dirofilaria*, *Babesia* and *Ehrlichia* species or leptospires, or with OspA, whole-cell, or other vaccines (Liang and others 2000b, O'Connor and others 2004).

In Europe a commercial immunoassay for antibodies to the C6 antigen (the C6 rapid immunoassay) has been used in one epidemiological study in Germany. It was found that the prevalence of antibodies to *B burgdorferi* in a random group of dogs analysed by a 4Dx SNAP-Test (IDEXX Laboratories) was 7.7 per cent, somewhat lower than the prevalence in healthy dogs of breeds other than the Bernese mountain dog in Switzerland (15 per cent) (Gerber and others 2007, Krupka and others 2007). However, no comparisons with conventional tests were made.

The aim of this study was to compare the results of tests for Lyme borreliosis in dogs from Switzerland by a conventional whole-cell ELISA in combination with a Western blot assay and the single Western blot bands, and by the C6 rapid immunoassay.

Materials and methods

Samples of serum were taken from 53 healthy Bernese mountain dogs and 30 healthy control large-breed dogs with long hair. They were tested for antibodies to *B burgdorferi* and retested after two-and-a-half to three years (Gerber and others 2007, 2009). The dogs were chosen at random. Owners were contacted and volunteered to join the study after a call from the Swiss Club for Bernese mountain dogs and the Swiss Newfoundland and Landseer club. Others were contacted directly if it was known that they owned a dog eligible for the study. The samples were collected between July 2002 and April 2003, and between June and October 2005.

Dogs were included in the study if they were more than four months old at the first examination, were healthy according to the owners, and had no obvious signs of a specific disease evaluated by a complete blood count, a serum biochemical analysis and urinalysis. The biochemical analysis included measurements of the concentrations of bilirubin, glucose, urea, creatinine, total protein, albumin, cholesterol, sodium, potassium, chloride, calcium and phosphorus and the activity of alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase and amylase. The urinalysis included a test strip (Combur-Test; Roche Diagnostics), microscopical examination of sediment and the determination of specific gravity and protein:creatinine ratio.

Serological testings

The samples were stored at -80°C until they were tested. An in-house ELISA for the detection of IgG antibodies to *B burgdorferi* sensu lato in canine blood serum was used (Wittenbrink and others 1996, Gerber and others 2007). Western blot examinations for the detection of IgG antibodies to *B burgdorferi* were also made in all but one dog with a negative ELISA, using a commercial test kit adapted for dogs (Virion). The tests were made according to the manufacturer's instructions and consisted of Western blot strips with defined partial antigens of *B burgdorferi* sensu stricto and *B afzelii*. In preliminary tests with positive and negative dog sera a dilution of 1:200 and a 1:2000 dilution of alkaline-phosphatase-rabbit-anti-dog IgG, H+L as conjugate (Sigma) were considered adequate. The Western blot results were interpreted according to criteria recommended for three European species of *B burgdorferi* sensu lato (Hauser and others 1997). Samples were considered positive if bands at the level of the partial antigens p100, p58, OspC, p21 or p18 were identified or if at least two bands at the level of the partial antigens p45, bmpa and p30 were identified. Bands at the level of the partial antigens OspB, OspA, OspD, p22 and OspE were considered too unspecific.

Samples were considered positive for antibodies to *B burgdorferi* in the combined tests if both the ELISA and the Western blot were positive.

A commercially available in-office C6 rapid immunoassay kit was used to detect antibodies to the IR6 region of the surface protein VlsE (SNAP 3Dx; IDEXX). This test also detects antibodies to *Ehrlichia canis* and *Dirofilaria immitis* antigen. The test was made according to the manufacturer's instructions. The test results were either positive or negative. All the tests for *E canis* and *D immitis* were negative.

TABLE 1: Levels of agreement between a combination of whole-cell ELISA and Western blot assay and a commercial immunoassay for antibodies to the C6 antigen of *Borrelia burgdorferi* in 53 Bernese mountain dogs and 30 control large-breed longhair dogs

Group	Agreement (%)	Kappa	P
All dogs	79	0.571	<0.001
Bernese mountain dogs	81	0.681	<0.001
Control dogs	75	0.347	0.01

TABLE 2: Sensitivity, specificity and accuracy of the commercial immunoassay for antibodies to the C6 antigen of *Borrelia burgdorferi* compared with a conventional ELISA in combination with a Western blot assay

C6 rapid immunoassay	All the dogs		Bernese mountain dogs		Control dogs	
	Positive	Negative	Positive	Negative	Positive	Negative
Positive	56	21	49	18	7	3
Negative	13	69	2	35	11	34
Sensitivity (%)		81		96		39
Specificity (%)		77		66		92
Accuracy (%)		79		81		75

Statistical analysis

The data were recorded and analysed by using a commercial computer program (Statistical Package for the Social Sciences for Windows version 11; SPSS). The agreement between the tests was evaluated by kappa statistics. If kappa values were from 0 to 0.2 the agreement between tests was considered poor, from 0.2 to 0.4 fair, from 0.4 to 0.6 moderate, from 0.6 to 0.8 good and from 0.8 to 1 almost perfect. The effects of age on the variables in the Bernese mountain dogs and the control dogs were compared by using the Mann-Whitney U test and the effects of gender were evaluated by Fisher's exact test. Differences were considered significant at $P<0.05$.

Results

One hundred and four samples were collected from the 53 Bernese mountain dogs and 55 samples from the 30 control dogs; 51 of the Bernese mountain dogs were sampled twice, 51 in the first sampling period and 53 in the second; 25 of the control dogs were sampled twice, 25 in the first sampling period and 30 in the second. When they were sampled the age of the dogs ranged from one to 11 years (median five years). The control dogs were significantly older than the Bernese mountain dogs (median six years v five years; $P=0.001$). There were 23 male (five neutered) and 60 female dogs (23 neutered) and the gender distribution was not significantly different between the Bernese mountain dogs and the control dogs. In the control group there were 14 Landseer, nine Newfoundland, two flat-coated retrievers, one golden retriever, one Belgian shepherd dog, two mixed-breed dogs and one Tibetan mastiff. The second samples were collected from the Bernese mountain dogs between 907 and 1113 days (median 1021 days) after the first, and between 899 and 1019 days (median 969 days) after in the control dogs.

Antibodies to *B burgdorferi*

The agreements between the tests are shown in Table 1. Considering all the samples together there was a significant ($P<0.001$) agreement between the C6 rapid immunoassay and the combination of the other tests (kappa=0.571). However, the agreement between the tests in the samples from the Bernese mountain dogs (kappa=0.681) was better than in the control dogs (kappa=0.347).

Sixty-nine (43 per cent) of the serum samples were considered positive taking the whole-cell ELISA combined with the Western blot assay as gold standard; the other 90 were negative. This seroprevalence does not indicate the true prevalence in the population because the samples were chosen randomly from a serum bank. The sensitivity, specificity and accuracy of the C6 rapid immunoassay in relation to the gold standard whole-cell ELISA combined with Western blot are shown in Table 2. In the samples from the first sampling period, the agreement

TABLE 3: Levels of agreement between the commercial immunoassay for antibodies to the C6 antigen of *Borrelia burgdorferi* and specific bands in the Western blot assay

Western blot band	Agreement (%)	Kappa	P	Western blot criteria
p100	84	0.686	<0.001	Western blot was considered positive if one of these bands was positive
p58	72	0.437	<0.001	
OspC	67	0.323	<0.001	
p21	59	0.116	0.001	
p18	53	0.040	0.111	
BmpA	65	0.285	<0.001	Western blot was considered positive if two of these bands were positive
p45	58	0.147	0.001	
p30	58	0.150	0.009	
OspD	82	0.633	<0.001	Bands considered unspecific
p22	61	0.203	0.001	
OspE	61	0.200	<0.001	
OspB	55	0.084	0.114	
OspA	54	0.055	0.157	

was good ($\kappa=0.679$; $P<0.001$) and in the second sampling period it was less good but still significant ($\kappa=0.474$; $P<0.001$). In the first sampling period the sensitivity of the C6 rapid immunoassay was 82 per cent and its specificity 86 per cent; in the second sampling period its sensitivity was 81 per cent and its specificity was 68 per cent.

Three of the Bernese mountain dogs and four of the control dogs had been vaccinated against Lyme borreliosis, but it was not known when. One of the Bernese mountain dogs and two of the control dogs were sampled twice, giving a total of 10 samples; of these four were positive in the conventional test combination but negative in the C6 rapid immunoassay; one sample was negative in the conventional test but positive in the C6 rapid immunoassay, and the other five samples gave the same results in both test systems (three positive and two negative). Of the 10 samples from the vaccinated dogs one was positive for OspA (one Bernese mountain dog) and in the C6 rapid immunoassay; two were positive for OspB (one Bernese mountain dog and one control dog) and both of them were positive in the C6 rapid immunoassay.

The agreements between the C6 rapid immunoassay and the occurrence or absence of bands in the Western blot assay were calculated (Table 3). The agreement between the occurrence of Western blot bands and the C6 rapid immunoassay was good for p100 and OspD, moderate for p58, OspC, BmpA and p22, poor but significant for p21, p45, p30 and OspE, and poor but not significant for p18, OspB and OspA.

Discussion

The C6 rapid immunoassay gave the same results as a combination of Western blot assay with a whole-cell ELISA in 79 per cent of the samples tested. A higher agreement rate has been observed in three studies from the USA; in one, with the same C6 rapid immunoassay kit, its results corresponded 100 per cent with results of an ELISA in combination with a Western blot in 18 dogs (Levy and others 2002); in another, the results of a C6 ELISA agreed 91 per cent with the results of a combination of a kinetic ELISA and Western blot examinations (Liang and others 2000b), and in a third an agreement rate of 93 per cent was reported with the C6 rapid immunoassay kit used in the present study (Goldstein and others 2007). All these studies were made in the USA where it is well established that there are different species of *B. burgdorferi* from those in Europe (Greene and Straubinger 2006), and this species difference might explain the lower agreement in the present study.

Different species also lead to different interpretation criteria for the tests. The criteria for the Western blot used in this study were based not only on the fact that European *Borrelia* species are different from those in the USA, but also on the fact that the antibody responses of human patients in Europe were less pronounced than in patients in the USA (Hauser and others 1997). Furthermore, less stringent criteria were used in human beings in Europe, owing to the occurrence of different genospecies in different areas. The criteria used in dogs in the present study were also less stringent than in one study on dogs in the USA, where

the Western blot was only considered positive if two or more bands were present in the 14 kD to 30 kD regions (Levy and others 2002). A comparison of the bands used for the decision in this study with the C6 rapid immunoassay showed that agreement was variable. The best agreement was found with bands at p100 and p58, two bands that were considered important for the diagnosis of Lyme borreliosis with IgG blots in people in Europe (Robertson and others 2000). This implies that the C6 rapid immunoassay used in this study is able to detect European strains of *B. burgdorferi*. The agreement between the C6 rapid immunoassay results and a band at the level of OspD (29/28 kD) was high. However, this band was not used for the diagnosis of *B. burgdorferi* antibodies because it was not found in the reference publication from Germany, from which the criteria for the Western blot interpretation were taken (Hauser and others 1997). Nevertheless, in one study of Swiss human patients, sera with a preferential reactivity to *B. garinii*, which is the most common genospecies in Switzerland, showed high reactivity to OspD (Ryffel and others 1999). This finding underlines the possibility of regional differences. In human medicine it was even suggested that in Europe immunoblotting should be regarded as an additional test, with an emphasis on specificity, which does not confirm but only supports the diagnosis of Lyme borreliosis (Robertson and others 2000).

The C6 rapid immunoassay is considered capable of differentiating between vaccinated and unvaccinated dogs. In this study too few of the dogs had been vaccinated, probably with a whole-cell vaccine, for this to be assessed. Nevertheless, fewer samples from the vaccinated dogs were found positive by the C6 rapid immunoassay than by the conventional tests. In the Western blot only three of the 10 samples from the vaccinated dogs had a band corresponding to *B. burgdorferi* OspA or OspB, bands which have been found more often in vaccinated dogs than in unvaccinated dogs (Gauthier and others 1999, Guerra and others 2000, Levy and others 2002). One explanation could be that the dogs had been vaccinated a long time ago. However, when the dogs were vaccinated was not known, and it is not known for how long antibodies can be detected after vaccination (Greene and Straubinger 2006).

In human patients with clinical Lyme disease in Europe, a sensitivity of 83 per cent and a specificity of 95 per cent were observed with a C6 ELISA (Liang and others 2000a). Ideally the C6 rapid immunoassay should also have been applied to clinically ill dogs. However, apart from lameness and fever, no other clinical signs could be induced in experimentally infected dogs (Greene and Straubinger 2006) and Lyme disease is probably over-diagnosed in dogs. For example, Speck and others (2007) found that in 44 per cent of dogs in which borreliosis was suspected on the basis of clinical signs another diagnosis was made after further investigations. Furthermore, it is well known that false-positive serological results can be due to cross-reactions with other bacteria which might belong to the natural microflora of dogs (Magnarelli and others 2000). The 13 dogs that did not react with the C6 rapid immunoassay but were positive in the other tests could have had cross-reactions with other bacteria in the whole-cell ELISA and Western blot assay. These results indicate that the positive predictive value of serological tests for Lyme borreliosis is low. The agreement between the tests was lower in the second samples than in the first. One reason could be that all the second samples were taken during the tick season whereas only some of the first samples were taken in the tick season. During a tick season samples might be taken shortly after infection, and C6 antibodies were found to increase earlier than antibodies detected by the whole-cell ELISAs (Liang and others 2000b, Straubinger and others 2000). This might have led to more positive tests in the C6 rapid immunoassay and therefore a low specificity in relation to the gold standard whole-cell ELISA combined with Western blot.

There were differences between the level of agreement between the tests in the Bernese mountain dogs and the control dogs, the agreement in the Bernese mountain dogs being better than in the control dogs. This difference is difficult to explain. One possible explanation would be the stronger reaction of Bernese mountain dogs to *B. burgdorferi* reported by Gerber and others (2007). It was found that positive control dogs had lower optical densities in the conventional ELISA than Bernese mountain dogs, and might therefore have been diagnosed negative in the C6 rapid immunoassay, which would have given the impression of

a low sensitivity, whereas the strong reaction of the negative Bernese mountain dogs would have led to a rather low specificity in this group. Differences in the reaction against different *B burgdorferi* strains have been reported and were used as an explanation for the different seroreactivity between European and North American patients. However, differences within one species is so far unique to Bernese mountain dogs (Gerber and others 2007).

The agreement between the C6 rapid immunoassay and the conventional diagnosis of *B burgdorferi* antibodies with a combination of Western blot assay and whole-cell ELISA was moderate ($\kappa=0.571$) but significant, suggesting that the C6 rapid immunoassay might also be used in the diagnosis of antibodies to *B burgdorferi* in Europe. However, there are interactions between the breed of the dogs and the results of the test that need further clarification.

Acknowledgements

The authors thank the owners who volunteered to join the study after a call from the Swiss Club for Bernese mountain dogs. The work was supported by the Swiss Club for Bernese mountain dogs, the Albert Heim Foundation and Novartis Animal Health Switzerland. C6 rapid immunoassay kits were provided by the Provet AG Lyssach.

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Chapter 7

The dilemma with Lyme borreliosis in the dog with particular consideration of „Lyme nephritis“ [Das Dilemma der Lyme Borreliose beim Hund unter besonderer Berücksichtigung der „Lyme Nephritis“. *In German*]

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Schweizer Archiv für Tierheilkunde 2009: 151(10); 479-483.

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Das Dilemma der Lyme Borreliose beim Hund unter besonderer Berücksichtigung der „Lyme Nephritis“

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Zusammenfassung

Lyme Borreliose ist die am häufigsten beschriebene, durch Zecken übertragene Infektionskrankheit des Menschen in der nördlichen Hemisphäre. Auch beim Hund werden gewisse Erkrankungen mit Lyme Borreliose in Zusammenhang gebracht, aber es konnten nur intermittierende Lahmheit mit geschwollenen Gelenken, Lymphadenomegalie, Fieber und Anorexie im Experiment nachgewiesen werden. Borreliose gilt als überdiagnostizierte Erkrankung. Der Begriff „Lyme Nephritis“ wurde für Hunde verwendet mit charakteristischen Nierenveränderungen und typischen klinischen Zeichen, bei denen Antikörper gegen *Borrelia burgdorferi* festgestellt wurden. In verschiedenen Untersuchungen wurde versucht, einen Zusammenhang zwischen Nierenerkrankung und einer *B. burgdorferi* Infektion zu zeigen, was aber bisher nicht gelang. Die Gründe für die Unklarheit über die Auswirkungen von Borrelien beim Hund liegen im häufigen Vorkommen von Antikörpern gegen *B. burgdorferi* sowie in der unklaren klinischen Präsentation und den ungenauen serologischen Tests.

Schlüsselwörter: Berner Sennenhund, *Borrelia burgdorferi*, Glomerulonephritis

The dilemma with Lyme borreliosis in the dog with particular consideration of „Lyme nephritis“

Lyme borreliosis is the most commonly reported tick-transmitted infectious disease in the northern hemisphere in humans. Certain diseases are associated with Lyme borreliosis in the dog as well, but only intermittent lameness with articular swelling, lymphadenomegaly, fever, and anorexia were experimentally documented. Lyme borreliosis is considered an over diagnosed disease. The term „Lyme nephritis“ was introduced for dogs with characteristic renal lesions and typical clinical signs, in which antibodies against *Borrelia burgdorferi* were found. Different studies have been aimed at showing a relation between renal disease and *B. burgdorferi* infection; however, this was not possible until now. Reasons for the uncertainty of the effects of *B. burgdorferi* in the dog are the high prevalence of circulating antibodies, the unspecific clinical picture and the inaccuracy of serologic tests.

Keywords: Bernese, *Borrelia burgdorferi*, Glomerulonephritis

Einleitung

Lyme Borreliose ist die am häufigsten beschriebene, durch Zecken übertragene Infektionskrankheit des Menschen in der nördlichen Hemisphäre. Schon am Anfang des 20. Jahrhunderts wurde beim Mensch das „Erythema migrans“ eine typische, flächenhafte, sich ringförmig ausbreitende Hautrötung in Zusammenhang mit Zeckenstichen beschrieben. Später in den 70er Jahren wurde bei Kindern in der Gegend der Ortschaft Lyme beobachtet, dass sich nach dem „Erythema migrans“ eine Arthritis entwickelte. Dieses klinische Bild wurde „Lyme Erkrankung“ genannt. Erst 1982 wurde der Erreger *Borrelia burgdorferi* als Verursacher der Erkrankung entdeckt

(Bratton et al., 2008). Auslöser der Lyme Borreliose sind *B. burgdorferi* sensu lato von denen die wichtigsten Arten *B. burgdorferi* sensu stricto, *B. garinii* und *B. afzelii* sind. In den USA kommt nur die Spezies *B. burgdorferi* sensu stricto vor, während in Europa *B. afzelii* und *B. garinii* vorherrschen (Hubálek und Halouzka, 1997). Der Erreger wird durch Zecken der Gattung Ixodes übertragen. Diese Zecken befallen Menschen und andere Wirbeltiere, Hunde eingeschlossen (Greene und Straubinger, 2006). Borreliose gilt sowohl beim Mensch als auch beim Hund als überdiagnostizierte Erkrankung. So hat eine kürzlich durchgeführte Untersuchung (Speck et al., 2007) gezeigt, dass 44 % der Hunde, mit klinischem Verdacht auf Borreliose eine andere Erkrankung hatten.

480 Originalarbeiten

Borreliose beim Hund

Während beim Mensch die Beziehung des Erregers *B. burgdorferi* zur „Lyme Erkrankung“ klar erscheint, besteht beim Hund weiterhin Unsicherheit über die Auswirkungen von Borrelien (Evison et al., 2006). Die einzigen experimentellen Studien, bei denen eindeutige klinische Symptome erzeugt werden konnten, wurden an jungen, mit *B. burgdorferi* infizierten Beagles in den USA durchgeführt. Die wichtigsten klinischen Symptome waren Fieber, intermittierende Lahmheit mit geschwollenen Gelenken, Lymphadenomegalie und Anorexie (Greene und Straubinger, 2006). Ein „Erythema migrans“ konnte nicht festgestellt werden. Bei den erwähnten Beagles wurde 49 bis 581 Tage nach der Infektion eine Sektion durchgeführt (Summers et al., 2005). Dabei wurden nur vergrößerte Lymphknoten mit follikulärer und parafollikulärer Hyperplasie, Polyarthritiden mit zum Teil makroskopisch geschwollenen Gelenken, eine Dermatitis in der Region, wo die Zecken angesetzt wurden und bei einigen Hunden Periarteritis und Perineuritis in Gelenkscapseln, perinodalen Weichteilen und der Haut festgestellt. In keinen anderen Organen wurden Veränderungen gefunden, insbesondere auch keine Nierenveränderungen. Experimentell wurde bisher nur eine einzige Studie mit in Europa vorkommenden Spezies durchgeführt (Jenal, 2002). In dieser Schweizer Studie serokonvertierte nur einer von 6 Hunden, bei denen Zecken angesetzt wurden und nur 4 Tiere zeigten vorübergehende Lahmheit.

Andere beim Hund beobachtete Symptome, die mit Lyme Borreliose in Verbindung gebracht wurden, sind rheumatoide Arthritis, neurologische Dysfunktionen, Herzarrhythmien und Glomerulonephritis und nur deshalb, weil gleichzeitig ein positiver Antikörpertiter gegen *B. burgdorferi* mit Lyme Borreliose vorhanden war. Im Experiment konnten diese klinischen Bilder jedoch nie erzeugt werden.

Diagnostik

Bedingt durch die unspezifischen klinischen Zeichen beim Hund ist die Diagnose der Lyme Borreliose schwierig (Littman et al., 2006). Zum Nachweis einer Infektion mit *B. burgdorferi* werden meist Antikörper gesucht. Leider trägt aber die fehlende Genauigkeit serologischer Tests viel zur Verunsicherung in der Diagnostik bei. Wegen der Ähnlichkeit der Antigene von *B. burgdorferi* mit denjenigen anderer Spirochäten und anderer Bakterien, die zur natürlichen Mikroflora der Hunde gehören oder als pathogene Keime vorkommen, sind falsch positive Resultate insbesondere bei Ganzzell-ELISA Tests häufig. Deshalb wird empfohlen, positive Ganzzell-ELISA Testresultate mittels Westernblot zu bestätigen (Magnarelli et al., 2000). Im Westernblot wird getestet, ob das Serum auf ausgewählte *B. burgdorferi* Antigene reagiert. Damit können durch kreuzreagierende Antikörper erzeugte, falsch

positive Resultate im Ganzzell-ELISA erkannt werden (Greene und Straubinger, 2006). Zudem lassen sich mittels Westernblot infizierte von geimpften Tieren unterscheiden. Da in verschiedenen Labors meist verschiedene Testverfahren durchgeführt werden, sind diese oft nicht vergleichbar.

Bei neueren Tests wird die Spezifität von ELISA Verfahren erhöht, indem das synthetische Peptid C6 als Antigen verwendet wird. Dieses Peptid entspricht der IR6, einer immunodominante Region des VlsE (Vmp-like sequence, Expressed) Oberflächen-Lipoproteins von Borrelien und ist genetisch, strukturell und antigenetisch bei verschiedenen *B. burgdorferi* Spezies konserviert (Greene und Straubinger, 2006). Die Gene der IR6 werden nur bei einer aktiven Infektion exprimiert. Aus diesem Grund werden stark positive Tests mit dem C6 ELISA als Zeichen einer aktiven Infektion interpretiert. In einer Studie (Levy et al., 2002) konnte gezeigt werden, dass bei Hunden mit einem C6-ELISA die gleichen Resultate erzielt wurden, wie mit einem Ganzzell-ELISA kombiniert mit einem Westernblot. Der C6-ELISA hatte gegenüber anderen ELISA Tests den Vorteil, dass bei geimpften Tieren keine positiven Reaktionen entstanden. Ein C6 Schnelltest hat sich auch bei Seren von Schweizer Hunden als brauchbar erwiesen (Sensitivität 81 %, Spezifität 77 %), was nicht selbstverständlich ist, da diese Tests vor allem an Tieren mit *B. burgdorferi* sensu stricto Infektionen evaluiert wurden (Haug, 2006). Die C6 ELISAs sind vielversprechende Tests zum Nachweis von Antikörpern gegen *B. burgdorferi*, insbesondere auch, weil durch die breitere Nutzung desselben Antigens in der Serodiagnostik die Vergleichbarkeit der Laborresultate verbessert wird.

Kultur und PCR zur Suche nach *B. burgdorferi* Keimen werden eher selten verwendet. Kulturen werden nur in spezialisierten Labors durchgeführt und sind sehr aufwändig. Zudem ist durch die geringe Keimdichte die Wahrscheinlichkeit eines positiven Resultats gering. PCR ist besser zugänglich, ihre Spezifität für eine aktive Infektion wird aber als tief beurteilt, weil PCR nicht zwischen lebenden und abgestorbenen Organismen unterscheidet und trotz klinischer Heilung positiv bleiben kann (Hengge et al., 2003).

Antikörperprävalenz

In einer älteren Studie aus der Schweiz (Pfister et al., 1989) wiesen von 176 gesunden Hunden 9 % einen positiven Antikörper Titer gegen *B. burgdorferi* auf. In neueren Arbeiten (Eichenberger, 2005; Gerber et al., 2007) wurde eine Prävalenz von 15 % (62 Hunde ohne Berner Sennenhunde) festgestellt. Der Grund einer höheren Prävalenz in der späteren Studie ist am ehesten in den unterschiedlichen Testverfahren zu suchen. Es bestehen nämlich keine Hinweise, dass sich der Infektionsdruck in der Schweiz in den Jahren zwischen den beiden Studien verstärkt hätte. Bereits 1986 wurden in 5 %–34 % der I. ricinus Zecken

Das Dilemma der Lyme Borreliose beim Hund 481

Borrelien gefunden und keine der untersuchten Zeckenpopulationen war frei von Borrelien (Aeschlimann et al., 1986). Ähnliche Zahlen wurden auch in einer Untersuchung von 2004 aus der Westschweiz mit 9–47% infizierten Zecken publiziert (Jouda et al., 2004). Es konnte auch gezeigt werden, dass Zecken in allen Regionen der Schweiz mit Borrelien befallen waren, aber die Verteilung der Spezies unterschiedlich war. So konnten im Tessin *B. garinii*, aber nicht *B. afzelii* oder *B. burgdorferi* sensu stricto festgestellt werden (Jouda et al., 2003), während in der Region Interlaken 47% *B. garinii*, 29% *B. afzelii* und 24% *B. burgdorferi* sensu stricto gefunden wurden (Jouda et al., 2004). Im Wallis wiederum, war *B. burgdorferi* sensu stricto am häufigsten (57%), gefolgt von *B. garinii* (37%) und *B. afzelii* (7%) (Péter et al., 1995).

Eine sehr hohe Prävalenz von 58% wurde beim Berner Sennenhund (n = 160) festgestellt und war damit deutlich höher als 15% bei anderen Hunderassen (n = 62), die in denselben Gegenden und unter denselben Bedingungen gehalten wurden (Gerber et al., 2007). Der Grund für diese hohe Prävalenz bei Berner Sennenhunden ist unklar, könnte aber auf eine mögliche Rasseprädisposition hinweisen.

Lyme Nephritis bei Hunden

„Lyme Nephritis“ ist eine typische Erkrankung beim Hund und der Begriff wurde durch Dambach et al. (1992, 1997) in den 90er Jahren geprägt. Die Namensgebung basierte auf der Beobachtung von speziellen Nierenveränderungen bei Hunden, die Antikörper gegen *B. burgdorferi* aufwiesen. Die Läsionen wurden als Glomerulonephritis, diffuse tubuläre Nekrosen mit Regeneration und interstieller Entzündung beschrieben. Am häufigsten wurde eine membranproliferative Glomerulonephritis gefunden. Beim Menschen wurden nur 3 Fälle publiziert, bei denen eine Nierenerkrankung mit Borreliose in Zusammenhang gebracht wurde (Kelly et al., 1999; Kirmizis et al., 2004; Zachaus, 2008).

Das klinische Bild beim Hund wurde als rasch verlaufende, fatal endende glomeruläre Erkrankung beschrieben (Sanders et al., 1997). Labrador- und Golden Retriever waren am häufigsten erkrankt. Es wird vermutet, dass die Nierenschädigungen ein immunvermitteltes Phänomen darstellen und es konnte gezeigt werden, dass die Konzentration von *B. burgdorferi* induzierten zirkulierenden Immunkomplexen mit einem hohen Titer im ELISA-Test korrelierte (Goldstein und Atwater, 2006). Bereits in früheren Untersuchungen wurde vermutet, dass *B. burgdorferi* Nephropathien auslösen kann. Magnarelli et al. (1987) beschrieben 5 Hunde, die Nierenerkrankungen und gleichzeitig Antikörper gegen *B. burgdorferi* aufwiesen. Drei der 5 Hunde zeigten auch Lahmheit und bei einem Hund wurden mittels Immunfluoreszenz Spirochaeten in der Niere nachgewiesen. Nur ein Jahr später wurden bei einem Labrador, bei dem im Urin und in den

Nieren Borrelien vermutet wurden, ebenfalls Nierenläsionen beschrieben (Grauer et al., 1988). Der Hund wies einen stark positiven Antikörpertiter gegen *B. burgdorferi* auf. In einer weiteren Untersuchung (Reusch et al., 1994), bei der gehäuft Glomerulonephritis bei Berner Sennenhunden festgestellt und eine familiäre Ursache vermutet wurde, waren alle getesteten Hunde ebenfalls *B. burgdorferi* positiv.

Bei allen oben beschriebenen Fällen wurde eine Nierenerkrankung gleichzeitig mit einem positiven Antikörpertiter festgestellt. Es hat sich aber gezeigt, dass bei Hunden, insbesondere bei Berner Sennenhunden, die Prävalenz von Antikörpern sehr hoch sein kann (bis 58%) und damit ein gleichzeitiges Vorliegen beider Befunde ohne direkten Zusammenhang möglich ist. Auch neuere Untersuchungen konnten keinen Zusammenhang zwischen *B. burgdorferi* und Glomerulonephritis herstellen. Bei Hunden, bei denen auf Grund von Antikörpern gegen *B. burgdorferi* und typischen histologischen Nierenveränderungen ein Verdacht auf „Lyme nephritis“ bestand, wurde für die Suche nach Borrelien die Fluoreszenz-in-situ-Hybridisierung (FISH) eingesetzt (Hutton et al., 2008). In 2 von 21 Fällen konnten zwar Borrelien entdeckt werden, das Ergebnis wurde aber von den Autoren selbst in Frage gestellt, da nur je ein Organismus in den Nierentubuli entdeckt wurde und zu einer sicheren Diagnose der Nachweis mehrere Erreger notwendig ist.

Weil Proteinurie der wichtigste Marker einer Glomerulopathie ist, wurde sowohl bei gesunden Labrador- und Golden Retrievern als auch bei gesunden Berner Sennenhunden untersucht, ob bei Antikörper positiven Hunden die Ausscheidung von Eiweiss im Urin erhöht ist (Goldstein et al., 2007; Gerber et al. 2009). In diesen Untersuchungen wurden verschiedene Methoden zur Proteinebestimmung im Urin verwendet, doch bei keiner der Methoden konnte ein Unterschied in der Eiweissausscheidung zwischen Hunden mit und Hunden ohne Antikörper gegen *B. burgdorferi* festgestellt werden. Auch eine späte Auswirkung von *B. burgdorferi* auf die Gesundheit von Hunden konnte nicht nachgewiesen werden. Seropositive Hunde, die nach 2–3 Jahren erneut untersucht wurden, wiesen nicht häufiger Anzeichen von Glomerulopathie oder Lahmheit als seronegative Hunde auf (Gerber et al., 2009).

Schlussfolgerungen

Lyme Borreliose ist eine beim Hund überdiagnostizierte Erkrankung. Selbst im Experiment konnten durch *B. burgdorferi* nur milde, wenig spezifische Symptome erzeugt werden. Die Diagnose wird durch die hohe Prävalenz von Antikörpern, die auch bei gesunden Hunden vorkommen und ungenaue Testverfahren erschwert. Der Nachweis, dass „Lyme Nephritis“ eine durch *B. burgdorferi* verursachte Nephropathie ist, konnte bisher nicht erbracht werden und deshalb sollte dieser Begriff vermieden werden.

482 Originalarbeiten

Le dilemme de la borréliose de Lyme chez le chien et en particulier de la «néphrite de Lyme»

La borréliose de Lyme est la maladie infectieuse humaine transmise par les tiques la plus fréquemment décrite dans l'hémisphère nord. Certaines affections sont également mises en rapport avec elle chez le chien mais, expérimentalement, on n'a pu mettre en évidence que des boiteries intermittentes avec des articulations enflées, un grossissement des ganglions lymphatiques, de la fièvre et de l'anorexie. On considère que la borréliose est une maladie surdiagnostiquée. Le concept de néphrite de Lyme a été utilisé chez le chien en présence d'altérations typiques des reins accompagnée de signes cliniques évidents, dans lesquels des anticorps contre *Borrelia burgdorferi* ont été trouvés. Dans plusieurs examens, on a essayé de trouver une relation entre l'affection rénale et une infection à *B. burgdorferi* mais sans succès jusqu'à présent. Les raisons du manque de clarté de l'action des *Borrelia* chez le chien sont à chercher dans la présence fréquente d'anticorps contre *B. burgdorferi* ainsi que dans les présentations cliniques peu claires et les tests sérologiques inexacts.

Il dilemma della borelliosi di Lyme nel cane con particolare attenzione alla „nefrite di Lyme“

La borelliosi di Lyme è la malattia infettiva trasmissibile all'uomo dalle zecche, la più frequentemente descritta nell'emisfero Nord. Anche nel cane sono state dimostrate sperimentalmente che, certe malattie sono strettamente connesse alla borelliosi di Lyme come la zoppia intermittente con gonfiore delle articolazioni, la linfadenomegalia, la febbre e l'anoressia. La borelliosi è una malattia utradiagnosticata. Il termine „nefrite di Lyme“ viene utilizzato per i cani, nei quali sono stati rilevati degli anticorpi contro la *Borrelia burgdorferi* e si sono riscontrati modifiche renali caratteristiche e segni clinici tipici. Con varie analisi, si è cercata la correlazione tra le malattie renali e un'infezione da *B. burgdorferi* ma finora non vi sono stati risultati. I motivi per la confusione sugli effetti della *Borellia* nei cani si trovano nella mancanza di una chiara presentazione clinica, di inesatti test sierologici e nella frequente comparsa di anticorpi contro la *B. burgdorferi*.

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Das Dilemma der Lyme Borreliose beim Hund 483

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Manuskripteingang: 27. Januar 2009
Angenommen: 19. Mai 2009

Chapter 8 Summary and Conclusions

This study provided some interesting answers to questions raised in the introduction:

What is the seroprevalence of *B. burgdorferi* in Bernese Mountain dogs?

Fifty-eight per cent of healthy Bernese Mountain dogs had antibodies against *B. burgdorferi* (Chapter 2). The control dogs, which consisted of large long-haired dogs of other breeds, had a prevalence of 15%. The reasons for this difference are obscure. The Bernese Mountain dogs and control dogs lived under comparable conditions and thus, a difference in exposure to infected ticks was ruled out as a possible reason. One could speculate that the dark coat of the Bernese Mountain dog hampers detection and removal of ticks by owners, and thus promotes the transmission of *B. burgdorferi* to dogs of this breed, but a dark coat was not a risk factor for borreliosis in control dogs. The subpopulation of control dogs with a dark coat had a significantly lower seroprevalence of *B. burgdorferi* than Bernese Mountain dogs (28% versus 58%, $P=0.011$).

Vaccination against *B. burgdorferi* was also ruled out as a possible reason for the difference between the two groups; of the four vaccinated Bernese Mountain dogs, one was seronegative and three were seropositive for *B. burgdorferi*, and of the six vaccinated control dogs, three were seropositive and three were seronegative.

Cross-reaction with other bacteria may potentially interfere with serological tests. To minimize this risk, all tested serum samples were absorbed using a mixture of formalin-inactivated bacteria before the ELISA was carried out. In addition, all serum samples were subjected to a micro-agglutination test for leptospira antibodies. Fifty-four per cent of dogs had antibodies to leptospira, but these had no apparent effect on the seroprevalence of *B. burgdorferi*; there was no difference between dogs that were seropositive and seronegative for *B. burgdorferi* with respect to the occurrence of leptospira antibodies.

Because we were unable to identify a cause for the high seroprevalence of *B. burgdorferi* in Bernese Mountain dogs, we wondered whether there was a breed disposition; however, a breed disposition to increased antibody titres against infectious agents has not been reported in dogs.

The consequence of the high seroprevalence of *B. burgdorferi* is, that it is difficult to

establish a causal relationship between infection with *B. burgdorferi* and clinical disease, because of the likelihood that the two occurrences are coincidental.

Is the seroprevalence of *B. burgdorferi* associated with an increase in urinary protein excretion, which in turn could be used as a marker of glomerular disease in Bernese Mountain dogs?

Urinary protein concentrations in healthy but *B. burgdorferi*-seropositive Bernese Mountain dogs did not differ from those in seronegative Bernese Mountain dogs or from those in control dogs (Chapter 3). To detect very small urinary protein concentrations and to identify urinary protein patterns, a sensitive immunoassay for urinary albumin and a sodium dodecyl sulphate-agarose gel electrophoresis were employed, in addition to conventional laboratory methods, such as urine test strips and measurement of the urine protein-to-creatinine ratio. These sensitive methods failed to detect any changes in urinary protein excretion in *B. burgdorferi*-seropositive Bernese Mountain dogs. Similarly, a study involving young healthy Golden retrievers did not find an association between seroprevalence of *B. burgdorferi* and microalbuminuria (Goldstein et al., 2007).

Are *B. burgdorferi*-seropositive Bernese Mountain dogs predisposed to clinical signs characteristic of glomerulonephritis later in life?

There was no information about the long-term effects of *B. burgdorferi* infection in adult dogs so far. Of 83 dogs, (33 were *B. burgdorferi*-seropositive and 50 seronegative at the initial examination), 57 maintained their serological status, 11 seroconverted and 12 seroreverted after a period of 2.5 to 3 years, when they were examined a second time (Chapter 4). There was no difference between Bernese Mountain dogs and control dogs with respect to change of serological status. Eight owners (3 of Bernese Mountain dogs and 5 of control dogs) felt that the general health of their dog was abnormal at the second examination. Although these were very non-specific assessments, the findings could reflect subclinical disease; similar non-specific signs have also been associated with ‘chronic’ Lyme disease in humans (Feder et al., 2007). However, a relationship between abnormal general condition and demeanour and a titre to *B. burgdorferi* could not be confirmed. On the other hand, there was

an association between age and abnormal general condition; dogs with a normal general condition were younger than dogs with an abnormal general condition (median, 6 versus 10 years). Furthermore, there was no association between lameness, which is the classical sign of experimental Lyme disease, and the occurrence of antibodies to *B. burgdorferi*. As well, there was no difference between the prevalence of lameness at the first and second examinations neither in the seropositive dogs nor in the seronegative dogs. This confirms the findings of a previous study where the authors postulated that the serological status of healthy dogs with respect to *B. burgdorferi* does not allow one to predict the likelihood of future lameness of the animal (Levy and Magnarelli, 1992).

The serum concentrations of urea and creatinine, two important indicators of renal insufficiency, did not increase between the first and second examinations in both groups. On the other hand, the proportion of Bernese Mountain dogs with proteinuria, as measured with test strips, did increase, but the increase occurred in both seropositive and seronegative dogs.

In summary, there was no difference in the health status of dogs 2.5 to 3 years after they were examined serologically and found to be either seronegative or seropositive for *B. burgdorferi*.

Do Bernese Mountain dogs have lower complement C3 concentrations compared with dogs of other breeds?

The reason we measured complement C3 in serum of Bernese Mountain dogs and compared the concentrations with those of dogs of other breeds was because infection, as well as glomerulonephritis, have been associated with lowered complement C3 concentrations (Chapter 5). However, this study showed that in Bernese Mountain dogs, complement C3 concentrations were not decreased; in fact, for unknown reasons, complement C3 concentrations were higher than in dogs of other breeds. Therefore, there was no breed-associated decrease in complement C3 that could explain the simultaneous occurrence of *B. burgdorferi* antibodies and glomerulonephritis in Bernese Mountain dogs. Regarding the higher level of C3 it was speculated that in Bernese Mountain dogs a breed which is susceptible to histiocytic sarcomas, very active dendritic cells might contribute to the increased serum level of complement C3. Histiocytic sarcomas likely arise from dendritic cells and it is known that dendritic cells produce complement C3 in humans and mice.

Are test methods based on recombinant peptide C6 adequate for the detection of antibodies against *B. burgdorferi* in dogs in Europe?

A conventional test method (whole-cell ELISA combined with Western blot) was compared with a rapid test based on recombinant peptide C6 (Chapter 6). The C6 test method had only been evaluated in dogs in the USA, and therefore, it could not automatically be assumed that it also worked in Europe, where different *B. burgdorferi* species predominate. However, we showed that this test produces reliable results under European conditions, although the agreement with conventional test methods was not as good as in American studies. Using conventional methods as the gold standard, the C6 test method carried out in our clinic had a sensitivity of 0.81 and a specificity of 0.77, compared with sensitivities of 0.75 to 1.00 and specificities of 0.94 to 1.00 in American studies (Liang et al., 2000; Levy, 2002; Goldstein et al., 2007). Interestingly, we found that the agreement between conventional tests and the C6 rapid test depended on the breed of dogs tested; whereas in the Bernese Mountain dogs sensitivity was 0.96 and specificity 0.66 in control dogs sensitivity was 0.39 and specificity 0.92 (Kappa= 0.681; $P < 0.001$ in Bernese Mountain dogs and 0.347; $P = 0.01$ in control dogs). We were unable to explain this difference.

Conclusions

The present study did not reveal an association between seroprevalence of *B. burgdorferi* and clinical signs of glomerular disease in Bernese Mountain dogs. Therefore we doubt that an association between *B. burgdorferi* infection and glomerulonephritis exists (Chapter 7). The high seroprevalence of *B. burgdorferi* in the Bernese Mountain dog population suggests that the simultaneous occurrence of glomerulonephritis and borreliosis is coincidental and does not have a causal component.

The commonly encountered simultaneous occurrence of glomerulonephritis and seroprevalence of *B. burgdorferi* in Bernese Mountain dogs is not associated with a decreased complement C3 concentration.

A rapid test that is based on recombinant peptide C6 is useful for the detection of *B. burgdorferi* antibodies in dogs in Europe.

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Chapter 9 Acknowledgments [in German]

Mein Dank geht an

Frau Professor Claudia E Reusch, dafür dass Sie mir das Thema überlassen hat und mir die Möglichkeit gab, diese Arbeiten durchzuführen.

Herrn Professor Max M Wittenbrink für seine Hilfsbereitschaft und seine Tatkräftige Unterstützung.

Die Doktorandinnen Simone Eichenberger und Katharina Haug für ihre Arbeit und die angenehme Zusammenarbeit.

Die Hundebesitzer für die für ihre Bereitschaft an den Studien teilzunehmen.

Die Mitarbeitenden an der Klinik und im Labor, die mitgeholfen haben die Daten für diese Arbeiten zu generieren.

Die Albert-Heim-Stiftung für die finanzielle Unterstützung

Den Gesundheitsfonds des Schweizerischen Klubs für Berner Sennenhunde für die finanzielle Unterstützung

Novartis Tiergesundheit Schweiz für die finanzielle Unterstützung.

Meiner Frau Renate Herren-Gerber dafür, dass sie mich begleitet.

Meinen Söhnen Patrick und Stefan, dafür, dass sie da sind.